TITLE: PHASE-SENSITIVELY DETECTED REDUCED DIMENSIONALITY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY FOR RAPID CHEMICAL SHIFT ASSIGNMENT AND SECONDARY STRUCTURE DETERMINATION OF PROTEINS

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PHASE SENSITIVELY-DETECTED REDUCED DIMENSIONALITY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY FOR RAPID CHEMICAL SHIFT ASSIGNMENT AND SECONDARY STRUCTURE DETERMINATION OF PROTEINS

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[0001]　　　The present invention claims the benefit of U.S. Provisional Patent Application Serial No. 60/399,332, filed July 26, 2002, which is hereby incorporated by reference in its entirety. This invention arose out of research sponsored by the National Science Foundation (Grant No. MCB 0075773) and

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FIELD OF THE INVENTION

[0002]　　　The present invention relates to methods of using phase

15　　sensitively-detected reduced dimensionality nuclear magnetic resonance (NMR) spectroscopy for obtaining chemical shift assignment and for structure determination of proteins.

BACKGROUND OF THE INVENTION

20　　[0003]　　　The use of triple resonance (TR) nuclear magnetic resonance (NMR) experiments for the resonance assignment of polypeptide chains *via* heteronuclear scalar connectivities (Montelione et al., J. Am Chem. Soc., 111:5474–5475 (1989); Montelione et al., J. Magn. Reson., 87:183–188 (1989); Kay et al., J. Magn. Reson., 89:496–514 (1990); Ikura et al., Biochemistry,

25　　29:4659–8979 (1990); Edison et al., Methods Enzymol., 239:3–79 (1994)) is a standard approach which neatly complements the assignment protocol based on ^1H–^1H nuclear Overhauser effects (NOE) (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986)). In addition, triple resonance NMR spectra are highly amenable to a fast automated analysis (Friedrichs et al., J.

30　　Biomol. NMR, 4:703–726 (1994); Zimmerman et al., J. Biomol. NMR,

4:241–256 (1994); Bartels et al., J. Biomol. NMR, 7:207–213 (1996); Morelle et al., J. Biomol. NMR, 5:154–160 (1995); Buchler et al., J. Magn. Reson., 125:34–42 (1997); Lukin et al., J. Biomol. NMR, 9:151–166 (1997)), yielding the $^{13}C^{\alpha/\beta}$ chemical shifts at an early stage of the assignment procedure. This enables both the identification of regular secondary structure elements without reference to NOEs (Spera et al., J. Am. Chem. Soc., 113: 5490–5491 (1991)) and the derivation of (ϕ,ψ)-angle constraints which serve to reduce the number of cycles consisting of nuclear Overhauser enhancement spectroscopy (NOESY) peak assignment and structure calculation (Luginbühl et al., J. Magn. Reson., B 109:229–233 (1995)).

[0004]   NMR assignments are prerequisite for NMR-based structural biology (Wüthrich, NMR of Proteins and Nucleic Acid, Wiley:New York (1986)) and, thus, for high-throughput (HTP) structure determination in structural genomics (Rost, Structure, 6:259–263 (1998); Montelione et al., Nature Struct. Biol., 6:11–12. (1999); Burley, Nature Struc Biol., 7:932–934 (2000)) and for exploring structure-activity relationships (SAR) by NMR for drug discovery (Shuker et al., Science, 274:1531–1534 (1996)). The aims of structural genomics are to (i) explore the naturally occurring "protein fold space" and (ii) contribute to the characterization of function through the assignment of atomic resolution three-dimensional (3D) structures to proteins. It is now generally acknowledged that NMR will play an important role in structural genomics (Montelione et al., Nature Struc. Biol., 7:982–984 (2000)). The resulting demand for HTP structure determination requires fast and automated NMR data collection and analysis protocols (Moseley et al., Curr. Opin. Struct. Biol., 9:635–642 (1999)).

[0005]   The establishment of a HTP NMR structural genomics pipeline requires two key objectives in data collection. Firstly, the measurement time should be minimized in order to (i) lower the cost per structure and (ii) relax the constraint that NMR samples need to be stable over a long period of measurement time. The recent introduction of commercial cryogenic probes (Styles et al., J. Magn. Reson., 60:397–404 (1984); Flynn et al., J. Am Chem. Soc., 122:4823–4824 (2000)) promises to reduce measurement times by about a factor

of ten or more, and will greatly impact the realization of this first objective. Secondly, reliable automated spectral analysis requires recording of a "redundant" set of multidimensional NMR experiments each affording good resolution (which requires appropriately long maximal evolution times in all indirect dimensions). Concomitantly, it is desirable to keep the total number of NMR spectra small in order to minimize "interspectral" variations of chemical shift measurements, which may impede automated spectral analysis. Straightforward consideration of this second objective would suggest increasing the dimensionality of the spectra, preferably by implementing a suite of four- or even higher-dimensional NMR experiments. Importantly, however, the joint realization of the first and second objectives is tightly limited by the rather large lower bounds of higher-dimensional TR NMR measurement times if appropriately long maximal evolution times are chosen.

[0006]   Hence, "sampling limited" and "sensitivity limited" data collection regimes are distinguished, depending on whether the sampling of the indirect dimensions or the sensitivity of the multidimensional NMR experiments "per se" determines the minimally achievable measurement time. As a matter of fact, the ever increasing performance of NMR spectrometers will soon lead to the situation where, for many protein samples, the sensitivity of the NMR spectrometers do not constitute the prime bottleneck determining minimal measurement times. Instead, the minimal measurement times encountered for recording conventional higher-dimensional NMR schemes will be "sampling limited," particularly as high sensitivity cryoprobes become generally available. As structure determinations of proteins rely on nearly complete assignment of chemical shifts, which are obtained using multidimensional ^{13}C, ^{15}N, ^{1}H- TR NMR experiments (Montelione et al., J. Am Chem. Soc., 111:5474–5475 (1989); Montelione, et al., J. Magn. Reson., 87:183–188 (1989); Ikura et al., Biochemistry, 29:4659–8979 (1990)), the development of TR NMR techniques that avoid the sampling limited regime represents a key challenge for future biomolecular NMR methods development.

[0007]   Reduced dimensionality (RD) TR NMR experiments (Szyperski et al., J. Biomol. NMR, 3:127–132 (1993); Szyperski et al., J. Am. Chem. Soc., 115:9307-9308 (1993); Szyperski et al., J. Magn. Reson., B 105:188-191 (1994);

Brutscher et al., J. Magn. Reson., B 105:77-82 (1994); Szyperski et al., J. Magn. Reson., B 108: 197-203 (1995); Brutscher et al., J. Biomol. NMR, 5:202-206 (1995); Löhr et al., J. Biomol. NMR, 6:189-197 (1995); Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996); Szyperski et al., J. Magn. Reson., 28:228-232

5   (1997); Bracken et al., J. Biomol. NMR, 9:94-100 (1997); Sklenar et al., J. Magn. Reson., 130:119-124 (1998); Szyperski et al., J. Biomol. NMR, 11:387-405 (1998)), designed for simultaneous frequency labeling of two spin types in a single indirect dimension, offer a viable strategy to circumvent recording NMR spectra in a sampling limited fashion. RD NMR is based on a projection

10   technique for reducing the spectral dimensionality of TR experiments: the chemical shifts of the projected dimension give rise to a cosine-modulation of the transfer amplitude, yielding in-phase peak pairs encoding n chemical shifts in a n-1 dimensional spectrum (Szyperski et al., J. Biomol. NMR, 3:127–132 (1993); Szyperski et al., J. Am. Chem. Soc., 115:9307-9308 (1993)). As a key result, this

15   allows recording projected four-dimensional (4D) NMR experiments with maximal evolution times typically achieved in the corresponding conventional 3D NMR experiments (Szyperski et al., J. Biomol. NMR, 3:127–132 (1993); Szyperski et al., J. Am. Chem. Soc., 115:9307-9308 (1993); Szyperski et al.,. J. Magn. Reson. B 105:188-191 (1994); Szyperski et al., J. Magn. Reson., B 108:

20   197-203 (1995); Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996); Szyperski et al., J. Magn. Reson., 28:228-232 (1997); Bracken et al., J. Biomol. NMR, 9:94-100 (1997); Sklenar et al., J. Magn. Reson., 130:119-124 (1998); Szyperski et al., J. Biomol. NMR, 11:387-405 (1998)). Furthermore, axial coherences, arising from either incomplete insensitive nuclei enhanced by

25   polarization transfer (INEPT) or heteronuclear magnetization, can be observed as peaks located at the center of the in-phase peak pairs (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996)). This allows both the unambiguous assignment of multiple doublets with degenerate chemical shifts in the other dimensions and the identification of peak pairs by symmetrization of spectral

30   strips about the position of the central peak (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996); Szyperski et al., J. Biomol. NMR, 11:387-405 (1998)). Hence, observation of central peaks not only restores the dispersion of the parent, higher-dimensional experiment, but also provides access to reservoir of axial peak

magnetization (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996)).
Historically, RD NMR experiments were first designed to simultaneously recruit
both ^1H and heteronuclear magnetization (Szyperski et al., J. Am. Chem. Soc.,
118:8146-8147 (1996)) for signal detection, a feature that has also gained interest
for improving transverse relaxation-optimized spectroscopy (TROSY) pulse
schemes (Pervushin et al., Proc. Natl. Acad. Sci. USA, 94:12366-12371 (1997);
Salzmann et al., J. Am. Chem. Soc., 121:844-848 (1999); Pervushin et al., J.
Biomol. NMR, 12:345-348, (1998)). Moreover, RD two-spin coherence NMR
spectroscopy (Szyperski et al., J. Biomol. NMR, 3:127–132 (1993)) subsequently
also called zero-quantum/double-quantum (ZQ/DQ) NMR spectroscopy (Rexroth
et al., J. Am. Chem. Soc., 17:10389-10390 (1995)), served as a valuable radio-
frequency (r.f.) pulse module for measurement of scalar coupling constants
(Rexroth et al., J. Am. Chem. Soc., 17: 10389-10390 (1995)) and cross-correlated
heteronuclear relaxation (Reif et al., Science, 276:1230-1233 (1997); Yang et al.,
J. Am. Chem. Soc., 121:3555-3556 (1999); Chiarparin et al., J. Am. Chem. Soc.,
122:1758-1761 (2000); Brutscher et al., J. Magn. Reson., 130:346-351 (1998);
Brutscher, Concepts Magn. Reson., 122:207-229 (2000)).

[0008] Recently, a suite of RD $^{13}C,^{15}N,^1H$-triple resonance NMR
experiments was presented for rapid and complete protein resonance assignment
(Szyperski et al., Proc. Natl. Acad. Sci. USA, 99:8009-8014 (2002)). Even when
using short measurement times, these experiments allow one to retain the high
spectral resolution required for efficient automated analysis. "Sampling limited"
and "sensitivity limited" data collection regimes were defined, respectively,
depending on whether the sampling of the indirect dimensions or the sensitivity of
a multidimensional NMR experiments per se determines the minimally required
measurement time. It was shown that RD NMR spectroscopy is a powerful
approach to avoid the "sampling limited regime", i.e., a standard set of
experiments allows one to effectively adapt minimal measurement times to
sensitivity requirements. It would be advantageous to implement those RD NMR
experiments in a manner that allows frequency discrimination of the projected
chemical shifts.

[0009] The present invention is directed to achieving these objectives.

SUMMARY OF THE INVENTION

[0010]   The present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) HA,CA,(CO),N,HN nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having two consecutive amino acid residues, i-1 and i: (1) an α-proton of amino acid residue i-1, $^1H^\alpha_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^\alpha_{i-1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, where the chemical shift values of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ of amino acid residue i-1 are connected to the chemical shift evolutions of $^{15}N_i$ and $^1H^N_i$ of amino acid residue i, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^\alpha_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^\alpha)$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^1H^N_i$ in a direct time domain dimension, $t_3(^1H^N)$, and (2) to sine modulate the $^{13}C^\alpha_{i-1}$ chemical shift evolution in $t_1(^{13}C^\alpha)$ with the chemical shift evolution of $^1H^\alpha_{i-1}$. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with an anti-phase peak pair derived from the sine modulating, where (1) the chemical shift values of $^{15}N_i$ and $^1H^N_i$ are measured in two frequency domain dimensions, $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$, respectively, and (2) the chemical shift values of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^\alpha)$, by the frequency difference between the two peaks forming the anti-phase peak pair and the frequency at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of $^1H^\alpha_{i-1}$ in a phase sensitive manner.

[0011]   The present invention also relates to a method of conducting a reduced dimensionality three-dimensional (3D) H,C,(C-TOCSY-CO),N,HN

nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having two consecutive amino acid residues, i-1 and i: (1) aliphatic protons of amino acid residue i-1, $^{1}H^{ali}_{i-1}$; (2) aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}_{i-1}$; (3) a polypeptide
5   backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_i$, where the chemical shift values of $^{1}H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein
10   sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^{1}H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ of amino acid residue i-1 are connected to the chemical shift evolutions of $^{15}N_i$ and $^{1}H^{N}_i$ of amino acid residue i, under conditions effective (1) to generate a NMR signal encoding the chemical shifts of $^{13}C^{ali}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in two indirect time domain
15   dimensions, $t_1(^{13}C^{ali})$ and $t_2(^{15}N)$, respectively, and the chemical shift of $^{1}H^{N}_i$ in a direct time domain dimension, $t_3(^{1}H^{N})$, and (2) to sine modulate the chemical shift evolutions of $^{13}C^{ali}_{i-1}$ in $t_1(^{13}C^{ali})$ with the chemical shift evolutions of $^{1}H^{ali}_{i-1}$. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1)
20   the chemical shift values of $^{15}N_i$ and $^{1}H^{N}_i$ are measured in two frequency domain dimensions, $\omega_2(^{15}N)$ and $\omega_3(^{1}H^{N})$, respectively, and (2) the chemical shift values of $^{1}H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{ali})$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively,
25   where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of $^{1}H^{ali}_{i-1}$ in a phase sensitive manner.

[0012]   Another aspect of the present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},CO,HA$ nuclear magnetic resonance (NMR) experiment by measuring the chemical shift
30   values for the following nuclei of a protein molecule having an amino acid residue, i: (1) a β-proton of amino acid residue i, $^{1}H^{\beta}_i$; (2) a β-carbon of amino acid residue i, $^{13}C^{\beta}_i$; (3) an α-proton of amino acid residue i, $^{1}H^{\alpha}_i$; (4) an α-carbon

of amino acid residue i, $^{13}C^{\alpha}_i$; and (5) a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$, where the chemical shift values of $^1H^{\alpha}_i/^{13}C^{\alpha}_i$ and $^1H^{\beta}_i/^{13}C^{\beta}_i$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and

5   applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^{\alpha}_i$, $^1H^{\beta}_i$, $^{13}C^{\alpha}_i$, and $^{13}C^{\beta}_i$ are connected to the chemical shift evolution of $^{13}C'_i$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^{\alpha}_i$, $^{13}C^{\beta}_i$ and $^{13}C'_i$ in a phase sensitive manner in two indirect time domain

10   dimensions, $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{13}C')$, respectively, and the chemical shift value of $^1H^{\alpha}_i$ in a direct time domain dimension, $t_3(^1H^{\alpha})$, and (2) to sine modulate the chemical shift evolutions of $^{13}C^{\alpha}_i$ and $^{13}C^{\beta}_i$ in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^1H^{\alpha}_i$ and $^1H^{\beta}_i$, respectively. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived

15   from the sine modulating where (1) the chemical shift values of $^{13}C'_i$ and $^1H^{\alpha}_i$ are measured in two frequency domain dimensions, $\omega_2(^{13}C')$ and $\omega_3(^1H^{\alpha})$, respectively, and (2) (i) the chemical shift values of $^1H^{\alpha}_i$ and $^1H^{\beta}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs, and (ii) the

20   chemical shift values of $^{13}C^{\alpha}_i$ and $^{13}C^{\beta}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the anti-phase peak pairs, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift values of $^1H^{\alpha}_i$ and $^1H^{\beta}_i$ in a phase sensitive manner.

[0013]   A further aspect of the present invention relates to a method of

25   conducting a reduced dimensionality three-dimensional (3D) $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having an amino acid residue, i: (1) a β-proton of amino acid residue i, $^1H^{\beta}_i$; (2) a β-carbon of amino acid residue i, $^{13}C^{\beta}_i$; (3) an α-proton of amino acid residue i, $^1H^{\alpha}_i$; (4) an α-carbon

30   of amino acid residue i, $^{13}C^{\alpha}_i$; (5) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (6) a polypeptide backbone amide proton of amino

acid residue i, $^1\text{H}^N_i$, where the chemical shift values of $^1\text{H}^\alpha_i/^{13}\text{C}^\alpha_i$ and $^1\text{H}^\beta_i/^{13}\text{C}^\beta_i$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin

5 polarization transfer where the chemical shift evolutions of $^1\text{H}^\alpha_i$, $^1\text{H}^\beta_i$, $^{13}\text{C}^\alpha_i$, and $^{13}\text{C}^\beta_i$ are connected to the chemical shift evolutions of $^{15}\text{N}_i$ and $^1\text{H}^N_i$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}\text{C}^\alpha_i$, $^{13}\text{C}^\beta_i$ and $^{15}\text{N}_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}\text{C}^{\alpha/\beta})$ and $t_2(^{15}\text{N})$, respectively, and the chemical shift

10 value of $^1\text{H}^N_i$ in a direct time domain dimension, $t_3(^1\text{H}^N)$, and (2) to sine modulate the chemical shift evolutions of $^{13}\text{C}^\alpha_i$ and $^{13}\text{C}^\beta_i$ in $t_1(^{13}\text{C}^{\alpha/\beta})$ with the chemical shift evolutions of $^1\text{H}^\alpha_i$ and $^1\text{H}^\beta_i$, respectively. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{15}\text{N}_i$ and $^1\text{H}^N_i$ are

15 measured in two frequency domain dimensions, $\omega_2(^{15}\text{N})$ and $\omega_3(^1\text{H}^N)$, respectively, and (2) (i) the chemical shift values of $^1\text{H}^\alpha_i$ and $^1\text{H}^\beta_i$ are measured in a frequency domain dimension, $\omega_1(^{13}\text{C}^{\alpha/\beta})$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs, and (ii) the chemical shift values of $^{13}\text{C}^\alpha_i$ and $^{13}\text{C}^\beta_i$ are measured in a frequency domain

20 dimension, $\omega_1(^{13}\text{C}^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the anti-phase peak pairs, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift values of $^1\text{H}^\alpha_i$ and $^1\text{H}^\beta_i$ in a phase sensitive manner.

[0014]   The present invention also relates to a method of conducting a reduced dimensionality three-dimensional (3D) H,C,C,H-COSY nuclear magnetic

25 resonance (NMR) experiment by measuring the chemical shift values for $^1\text{H}^m$, $^{13}\text{C}^m$, $^1\text{H}^n$, and $^{13}\text{C}^n$ of a protein molecule where m and n indicate atom numbers of two CH, CH_2 or CH_3 groups that are linked by a single covalent carbon-carbon bond in an amino acid residue, where the chemical shift values of $^1\text{H}^m$ and $^{13}\text{C}^m$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase

30 sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effects a nuclear spin polarization transfer where the chemical shift evolutions of $^1\text{H}^m$ and $^{13}\text{C}^m$ are

connected to the chemical shift evolutions of $^1H^n$ and $^{13}C^n$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$, respectively, and the chemical shift value of $^1H^n$ in a direct

5   time domain dimension, $t_3(^1H^n)$, and (2) to sine modulate the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of ^1H_m. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{13}C^n$ and $^1H^n$ are measured in two frequency domain dimensions,

10   $\omega_2(^{13}C^n)$ and $\omega_3(^1H^n)$, respectively, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value

15   of ^1H_m in a phase sensitive manner.

[0015]   Another aspect of the present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) H,C,C,H-TOCSY nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for $^1H^m$, $^{13}C^m$, $^1H^n$, and $^{13}C^n$ of a protein molecule where m and n indicate

20   atom numbers of two CH, CH_2 or CH_3 groups that may or may not be directly linked by a single covalent carbon-carbon bond in an amino acid residue, where the chemical shift values of $^1H^m$ and $^{13}C^m$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the

25   protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^m$ and $^{13}C^m$ are connected to the chemical shift evolutions of $^1H^n$ and $^{13}C^n$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$, and the chemical shift value of $^1H^n$ in a direct time domain dimension, $t_3(^1H^n)$, and (2) to sine modulate

30   the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of $^1H^m$. Then, the NMR signals are processed to generate a sine-modulated 3D

NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{13}C^n$ and $^1H^n$ are measured in two frequency domain dimensions, $\omega_2(^{13}C^n)$ and $\omega_3(^1H^n)$, respectively, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$,

5   by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of ^1H_m in a phase sensitive manner.

[0016]   A further aspect of the present invention relates to a method of

10   conducting a reduced dimensionality two-dimensional (2D) <u>HB</u>,<u>CB</u>,(CG,CD),HD nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule: (1) a β-proton of an amino acid residue with an aromatic side chain, $^1H^\beta$; (2) a β-carbon of an amino acid residue with an aromatic side chain, $^{13}C^\beta$; and (3) a δ-proton of an amino acid

15   residue with an aromatic side chain, $^1H^\delta$, where the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ which are encoded in a peak pair of a 2D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^\beta$ and $^{13}C^\beta$ are

20   connected to the chemical shift evolution of $^1H^\delta$, under conditions effective (1) to generate NMR signals encoding the chemical shift value of $^{13}C^\beta$ in a phase sensitive manner in an indirect time domain dimension, $t_1(^{13}C^\beta)$, and the chemical shift value of $^1H^\delta$ in a direct time domain dimension, $t_2(^1H^\delta)$, and (2) to sine modulate the chemical shift evolution of $^{13}C^\beta$ in $t_1(^{13}C^\beta)$ with the chemical shift

25   evolution of $^1H^\beta$. Then, the NMR signals are processed to generate a sine-modulated 2D NMR spectrum with an anti-phase peak pair derived from the sine modulating where (1) the chemical shift value of $^1H^\delta$ is measured in a frequency domain dimension, $\omega_2(^1H^\delta)$, and (2) the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ are measured in a frequency domain dimension, $\omega_1(^{13}C^\beta)$, by the frequency difference

30   between the two peaks forming the anti-phase peak pair and the frequency at the center of the two peaks, respectively, where the sine-modulated 2D NMR

spectrum enables detection of the chemical shift value of $^1H^\beta$ in a phase sensitive manner.

[0017]   The present invention also relates to a method of conducting a reduced dimensionality two-dimensional (2D) $\underline{H},\underline{C},H$-COSY nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for $^1H^m$, $^{13}C^m$, and $^1H^n$ of a protein molecule where m and n indicate atom numbers of two CH, CH_2 or CH_3 groups in an amino acid residue, where the chemical shift values of $^1H^m$ and $^{13}C^m$ which are encoded in a peak pair of a 2D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^m$ and $^{13}C^m$ are connected to the chemical shift evolution of $^1H^n$, under conditions effective (1) to generate NMR signals encoding the chemical shift value of $^{13}C^m$ in a phase sensitive manner in an indirect time domain dimension, $t_1(^{13}C^m)$, and the chemical shift value of $^1H^n$ in a direct time domain dimension, $t_2(^1H^n)$, and (2) to sine modulate the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of $^1H^m$. Then, the NMR signals are processed to generate a sine-modulated 2D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift value of $^1H^n$ is measured in a frequency domain dimension, $\omega_2(^1H^n)$, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 2D NMR spectrum enables detection of the chemical shift value of ^1H_m in a phase sensitive manner.

[0018]   Another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^1H^\alpha$, an α-carbon, $^{13}C^\alpha$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1H^\alpha$ and $^{13}C^\alpha$ which are encoded in a peak pair of a 3D

NMR spectrum are detected in a phase sensitive manner, including: (1) a RD three-dimensional (3D) H̲A̲,C̲A̲,(CO),N,HN NMR experiment to measure and connect chemical shift values of the α-proton of amino acid residue i-1, $^1\text{H}^{\alpha}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}\text{C}^{\alpha}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}\text{N}_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1\text{H}^{\text{N}}_i$ and (2) a RD 3D HNNC̲A̲H̲A̲ NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i, $^1\text{H}^{\alpha}_i$, the α-carbon of amino acid residue i, $^{13}\text{C}^{\alpha}_i$, $^{15}\text{N}_i$, and $^1\text{H}^{\text{N}}_i$. Then, sequential assignments of the chemical shift values of $^1\text{H}^{\alpha}$, $^{13}\text{C}^{\alpha}$, ^{15}N, and $^1\text{H}^{\text{N}}$ are obtained by (i) matching the chemical shift values of $^1\text{H}^{\alpha}_{i-1}$ and $^{13}\text{C}^{\alpha}_{i-1}$ with the chemical shift values of $^1\text{H}^{\alpha}_i$ and $^{13}\text{C}^{\alpha}_i$, (ii) using the chemical shift values of $^1\text{H}^{\alpha}_{i-1}$ and $^{13}\text{C}^{\alpha}_{i-1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0019]   Yet another aspect of the present invention relates to a method for sequentially assigning chemical shift values of a β-proton, $^1\text{H}^{\beta}$, a β-carbon, $^{13}\text{C}^{\beta}$, an α-proton, $^1\text{H}^{\alpha}$, an α-carbon, $^{13}\text{C}^{\alpha}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1\text{H}^{\text{N}}$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1\text{H}^{\alpha/\beta}$ and $^{13}\text{C}^{\alpha/\beta}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D H̲$^{\alpha/\beta}$C̲$^{\alpha/\beta}$(CO)NHN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i-1, $^1\text{H}^{\beta}_{i-1}$, the β-carbon of amino acid residue i-1, $^{13}\text{C}^{\beta}_{i-1}$, the α-proton of amino acid residue i-1, $^1\text{H}^{\alpha}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}\text{C}^{\alpha}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}\text{N}_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1\text{H}^{\text{N}}_i$ and (2) a RD 3D H̲$^{\alpha/\beta}$,C̲$^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i, $^1\text{H}^{\beta}_i$, the β-carbon of amino acid

residue i, $^{13}C^{\beta}{}_{i}$, the α-proton of amino acid residue i, $^{1}H^{\alpha}{}_{i}$, the α-carbon of amino acid residue i, $^{13}C^{\alpha}{}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}{}_{i}$. Then, sequential assignments of the chemical shift values of $^{1}H^{\beta}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, and $^{1}H^{N}$ are obtained by (i) matching the chemical shift values of the α- and β-protons of amino acid residue i-1, $^{1}H^{\alpha/\beta}{}_{i-1}$, and the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$, with the chemical shift values of $^{1}H^{\alpha/\beta}{}_{i}$ and $^{13}C^{\alpha/\beta}{}_{i}$, (ii) using the chemical shift values of $^{1}H^{\alpha/\beta}{}_{i-1}$ and $^{13}C^{\alpha/\beta}{}_{i-1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0020] A further aspect of the present invention involves a method for sequentially assigning chemical shift values of aliphatic protons, $^{1}H^{ali}$, aliphatic carbons, $^{13}C^{ali}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^{1}H^{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^{1}H^{ali}$ and $^{13}C^{ali}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D H̲,C̲,(C-TOCSY-CO),N,HN NMR experiment to measure and connect the chemical shift values of the aliphatic protons of amino acid residue i-1, $^{1}H^{ali}{}_{i-1}$, the aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}{}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_{i}$, and the polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_{i}$ and (2) a RD 3D H̲$^{\alpha/\beta}$,C̲$^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i, $^{1}H^{\beta}{}_{i}$, the β-carbon of amino acid residue i, $^{13}C^{\beta}{}_{i}$, the α-proton of amino acid residue i, $^{1}H^{\alpha}{}_{i}$, the α-carbon of amino acid residue i, $^{13}C^{\alpha}{}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}{}_{i}$. Then, sequential assignments of the chemical shift values of $^{1}H^{ali}$, $^{13}C^{ali}$, ^{15}N, and $^{1}H^{N}$ are obtained by (i) matching the chemical shift values of the α- and β-protons of amino acid residue i-1, $^{1}H^{\alpha/\beta}{}_{i-1}$, and the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$, with the chemical shift values of $^{1}H^{\alpha/\beta}{}_{i}$ and $^{13}C^{\alpha/\beta}{}_{i}$ of amino acid residue i, (ii) using the chemical shift values of $^{1}H^{ali}{}_{i-1}$ and $^{13}C^{ali}{}_{i-1}$ to

identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

5   [0021]   The present invention also relates to a method for sequentially assigning chemical shift values of aliphatic protons, $^1H^{ali}$, aliphatic carbons, $^{13}C^{ali}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D H̲,C̲,(C-TOCSY-CO),N,HN NMR experiment to measure and connect the chemical shift values of the aliphatic protons of amino acid residue i-1, $^1H^{ali}_{i-1}$, the aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a RD 3D HNNC̲A̲H̲A̲ NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i, $^1H^\alpha_i$, the α-carbon of amino acid residue i, $^{13}C^\alpha_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}$, $^{13}C^{ali}$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of the α-proton of amino acid residue i-1, $^1H^\alpha_{i-1}$, and the α-carbon of amino acid residue i-1, $^{13}C^\alpha_{i-1}$, with the chemical shift values of $^1H^\alpha_i$ and $^{13}C^\alpha_i$, (ii) using the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0022]   Another aspect of the present invention involves a method for obtaining assignments of chemical shift values of ^1H, ^{13}C and ^{15}N of a protein molecule. The method involves providing a protein sample and conducting four reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of ^1H and ^{13}C which are

encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner and (1) a first experiment is selected from a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, a RD 3D $\underline{HA},\underline{CA}$,(CO),N,HN NMR experiment, or a RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment for obtaining sequential

5   correlations of chemical shift values; (2) a second experiment is selected from a RD 3D HNN$\underline{CAHA}$ NMR experiment, a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, or a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment for obtaining intraresidue correlations of chemical shift values; (3) a third experiment is a RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment for obtaining assignments of sidechain

10   chemical shift values; and (4) a fourth experiment is a RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment for obtaining assignments of aromatic sidechain chemical shift values.

[0023]   The present invention enables one to record an entire suite of RD NMR experiments used for obtaining chemical shift assignments and secondary

15   structure determination of a protein molecule in a manner that enables frequency discrimination for the projected chemical shift and allows one to place the two peaks forming a peak pair into separate spectra. The frequency discrimination allows one to place the radiofrequency carrier in the center of the projected chemical shift range.

20

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]   Figure 1 is a schematic presentation describing cosine-modulated and sine-modulated RD NMR data acquisition and processing, as well as the editing of the peaks forming RD NMR peak pairs into separate spectra. Data sets

25   (1) and (2) correspond to real part and imaginary part, respectively, of a cosine-modulated RD NMR experiment, in which Ω_y is detected in a phase sensitive manner and the evolution of the chemical shift Ω_x effects a cosine modulation. Data sets (3) and (4) correspond to real part and imaginary part, respectively, of a sine-modulated RD NMR experiment, in which Ω_y is detected in a phase sensitive

30   manner and the evolution of Ω_x effects a sine modulation, further enabling the phase sensitive detection of Ω_x. The two data sets as well as the corresponding

cosine and sine functions for the cosine-modulated RD NMR and sine-modulated RD NMR are shown in (A) and (B), respectively. Forming the sum and the difference of the cosine-modulated and sine-modulated RD NMR spectra allows one to edit the two peaks forming the peak pairs into separate spectra. The data sets that need to be linearly combined in the time domain to achieve such editing are shown in (C) and (D).

[0025] Figures 2A-K show the polarization transfer pathways (top) and stick diagrams of the in-phase peak pattern observed along $\omega_1(^{13}C)$ (bottom) for the cosine-modulated RD NMR experiments implemented for the present invention (the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN experiment, the 3D HACA(CO)NHN experiment, the 3D HC(C-TOCSY-CO)NHN experiment, the 3D HNNCAHA experiment, the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA experiment, the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN experiment, the 3D HNN<CO,CA> experiment, the 3D HCCH-COSY experiment, the 3D HCCH-TOCSY experiment, the 2D HBCB(CGCD)HD experiment, and the 2D ^{1}H-TOCSY-relayed-HCH-COSY experiment, respectively). For the sine-modulated RD NMR experiments, anti-phase peak pairs are observed (see Figure 1) instead of the in-phase peak pattern for the cosine-modulated RD NMR experiments. The boxes comprise nuclei whose chemical shifts are measured in the common dimension ω_1, and the nuclei which are detected in quadrature in t_1 are marked with an asterisk. Bold solid and hatched boxes indicate intraresidue and sequential connectivities, respectively, and the resulting signals sketched in the stick diagrams are represented accordingly. Those ^{13}C nuclei whose magnetization is used to detect central peaks (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996), which is hereby incorporated by reference in its entirety), as well as the resulting subspectrum II shown at the bottom, are highlighted in grey. The magnetization is frequency labeled with single-quantum coherence of the encircled nuclei during t_2 and detected on the boxed protons. Except for Figure 2G, the in-phase splittings $2\Delta\Omega(^{1}H)$ are equal to $2\kappa \cdot \delta\Omega(^{1}H)[\gamma(^{1}H)/\gamma(^{13}C)]$, where κ, $\delta\Omega(^{1}H)$ and $\gamma(X)$ denote the scaling factor applied for ^{1}H chemical shift evolution (set to 1.0 for the present study), the chemical shift difference with respect to the apparent ^{1}H carrier position, and the gyromagnetic ratio of nucleus X, respectively. In Figure 2G, the in-phase splittings $2\Delta\Omega(^{13}C^{\alpha})$ are equal to

$2\kappa \cdot \delta\Omega(^{13}C^{\alpha})$, where κ and $\delta\Omega(^{13}C^{\alpha})$ are the scaling factor applied for $^{13}C^{\alpha}$ chemical shift evolution[13] (set to 0.5 for the present study) and the chemical shift difference with respect to the apparent $^{13}C^{\alpha}$ carrier position, respectively.

[0026]   Figure 3A illustrates the experimental scheme for the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for ^{1}H chemical shift evolution during t_1 is set to 1.0. The high-power 90° pulse lengths were: 5.9 μs for ^{1}H, 15.4 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power, and ^{13}C decoupling during $t_1(^1H)$ is achieved using a $(90_x$-180_y-$90_x)$ composite pulse. Subsequently, the 90° and 180° pulse lengths applied for $^{13}C^{\alpha/\beta}$ are adjusted to 47.5 μs and 42.5 μs, respectively, to minimize perturbation of ^{13}CO spins. The width of the 90° pulse applied on ^{13}CO pulse is 52 μs and the corresponding 180° pulses are applied with same power. A SEDUCE 180° pulse with a length of 200 μs is used to decouple ^{13}CO during t_1 and τ_4. The length of the spin-lock purge pulses SL_x and SL_y are 1.2 ms and 0.6 ms, respectively. WALTZ16 is employed to decouple ^{1}H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during acquisition (r.f. = 1.78 kHz). The SEDUCE sequence is used for decoupling of $^{13}C^{\alpha}$ during ^{15}N evolution period (r.f. = 1.0 kHz). The ^{1}H r.f. carrier is placed at 0 ppm before the start of the semi constant time ^{1}H chemical shift evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^{1}H pulse. Initially, the ^{13}C and ^{15}N r. f. carriers are set to 43 ppm and 120.9 ppm, respectively. The ^{13}C carrier is set to 56 ppm during the second $\tau_4/2$ delay. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 μs, 16 G/cm); G3 (250 μs, 29.5 G/cm); G4 (250 μs, 30 G/cm); G5 (1.5 ms, 20 G/cm);G6 (1.25 ms, 30 G/cm); G7 (500 μs, 8 G/cm); G8 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 800 μs, τ_2 = 3.1 ms, τ_3 = 3.6 ms, τ_4 = 7.2 ms, τ_5 = 4.4 ms, τ_6 =

24.8 ms, τ_7 = 24.8 ms, τ_8 = 5.5 ms, τ_9 = 4.6 ms, τ_{10} = 1.0 ms. ^{1}H-frequency

labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) =

1 μs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 μs, Δt_1^b = 19.3 μs, Δt_1^c = -14 μs. Hence, the

fractional increase of the semi constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c$ /

5   Δt_1^a = 0.58. Phase cycling: ϕ_1 = x; ϕ_2 = x,x,-x,-x; ϕ_3 = x, -x; ϕ_4 = x, -x; ϕ_5 = x; ϕ_6

= x, x, -x, -x; ϕ_7 = x; ϕ_8 (receiver) = x, -x, -x, x. The sensitivity enhancement

scheme of Kay (Cavanagh et al., Protein NMR Spectroscopy, Academic Press,

San Diego, (1996), which is hereby incorporated by reference in its entirety) is

employed, i.e., the sign of G_6 is inverted in concert with a 180° shift of ϕ_7.

10   Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases

ϕ_2 and ϕ_5, respectively, according to States-TPPI (Cavanagh et al., Protein NMR

Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated

by reference in its entirety). For acquisition of central peaks derived from ^{13}C

steady state magnetization, a second data set with ϕ_1 = -x is collected. The sum

15   and the difference of the two resulting data sets generate subspectra II and I,

respectively, containing the central peaks and peak pairs.

[0027]   Figure 3B illustrates the experimental scheme for the 3D

HACA(CO)NHN experiment. Rectangular 90° and 180° pulses are indicated by

thin and thick vertical bars, respectively, and phases are indicated above the

20   pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied

along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for

^{1}H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse

lengths were: 5.8 μs for ^{1}H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C

prior to $t_1(^{13}C)$ are applied at high power, and ^{13}C decoupling during $t_1(^1H)$ is

25   achieved using a (90$_x$-180$_y$-90$_x$) composite pulse. Subsequently, the 90° and 180°

pulse lengths of $^{13}C^\alpha$ are adjusted to 51.5 μs and 46 μs, respectively, to minimize

perturbation of the ^{13}CO spins. The width of the 90° pulses applied to ^{13}CO pulse

is 52 μs and the corresponding 180° pulses are applied with same power. A

SEDUCE 180° pulse with a length 252 μs is used to decouple ^{13}CO during t_1. The

30   length of the spin-lock purge pulses SL_x and SL_y are 2.5 ms and 1 ms,

respectively. WALTZ16 is employed to decouple ^{1}H (r.f. field strength = 9.2

kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N

during acquisition (r.f. = 1.78 kHz). The SEDUCE sequence is used for decoupling of $^{13}C^\alpha$ during the ^{15}N chemical shift evolution period (r.f. = 1.0 kHz). The ^1H r.f. carrier is placed at 0 ppm before the start of the semi constant time ^1H evolution period, and then switched to the water line at 4.78 ppm after the second

5     $90°$ ^1H pulse. The $^{13}C^\alpha$ and ^{15}N r.f. carriers are set to 56.1 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 μs, 16 G/cm); G3 (1 ms, 24 G/cm); G4 (250 μs, 30 G/cm); G5 (1.5 ms, 20 G/cm); G6 (1.25 ms, 30 G/cm); G7 (500 μs, 8 G/cm); G8 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery

10   delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 1.6 ms, τ_2 = 3.6 ms, τ_3 = 4.4 ms, τ_4 = τ_5 = 24.8 ms, τ_6 = 5.5 ms, τ_7 = 4.6 ms, τ_8 = 1 ms. ^1H-frequency labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 μs, t_1^c (0) = 1.701 ms, Δt_1^a = 60 μs, Δt_1^b = 35.4 μs, Δt_1^c = -24.6 μs. Hence, the fractional increase of the semi

15   constant-time period with t_1 equals to λ = 1 + Δt_1^c / Δt_1^a = 0.58. Phase cycling: ϕ_1 = x; ϕ_2 = x, x, -x, -x; ϕ_3 = x, -x; ϕ_4 = x; ϕ_5 = x, x, -x, -x; ϕ_6 = x; ϕ_7(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) is employed., *i.e.*, the sign of G6 is

20   inverted in concert with a $180°$ shift of ϕ_6. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_2 and ϕ_4, respectively, according to States-TPPI (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second

25   data set with ϕ_1 = -x is collected. The sum and the difference of the two resulting data sets generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0028]   Figure 3C illustrates the experimental scheme for the 3D <u>HC</u>(C-TOCSY-CO)NHN experiment. Rectangular $90°$ and $180°$ pulses are indicated by

30   thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied

along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for ^{1}H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^{1}H and 15.5 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}$C) are applied at high power, and ^{13}C decoupling during $t_1(^1$H) is

5   achieved using a (90$_x$-180$_y$-90$_x$) composite pulse. Subsequently, the 90° and 180° pulse lengths applied for ^{13}C are adjusted to 47.0 μs and 42.5 μs, respectively, to minimize perturbation of ^{13}CO spins. The width of the 90° pulses applied to ^{13}CO pulse is 52 μs and the corresponding 180° pulses are applied with same power. A SEDUCE 180° pulse with a length 200 μs is used to decouple ^{13}CO during t_1 and

10   τ_4 period. WALTZ16 is employed to decouple ^{1}H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during acquisition (r.f. = 1.78 kHz). The SEDUCE sequence is used for decoupling of ^{13}C$^\alpha$ during the ^{15}N chemical shift evolution period (r.f. = 1.0 kHz). The ^{1}H r.f. carrier is placed at 0 ppm before the start of the semi constant time ^{1}H evolution

15   period, and then switched to the water line at 4.78 ppm after the second 90° ^{1}H pulse. The ^{13}C and ^{15}N r. f. carriers are set to 43 ppm and 120.9 ppm, respectively. The lengths of the ^{13}C spin-lock purge pulses, SL$_x$, are 2.5 ms and 1.25 ms, respectively, before and after the carbon-carbon total correlation spectroscopy (TOCSY) relay. ^{13}C isotropic mixing is accomplished using DIPSI-2 scheme with

20   a r.f. field strength of 8.5 kHz. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (2 ms, 30 G/cm); G2 (100 μs, 8 G/ cm); G3 (200 μs, 4 G/cm); G4 (2 ms, 30 G/cm); G5(1.25 ms, 30 G/cm); G6 (500 μs, 5 G/ cm); G7 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The

25   delays are: τ_1 = 950 μs, τ_2 = 3.1 ms, τ_3 = 3.6 ms, τ_4 = 7.2 ms, τ_5 = 4.45 ms, τ_6 = 24.8 ms, τ_7 = 24.8 ms, τ_8 = 5.5 ms, τ_9 = 4.8 ms, τ_{10} = 1 ms. ^{1}H-frequency labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 μs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 μs, Δt_1^b = 19.3 μs, Δt_1^c = -14 μs. Hence, the fractional increase of the semi constant-time period with t_1 equals to λ = 1 + Δt_1^c / Δt_1^a =

30   0.58. Phase cycling: ϕ_1 = x; ϕ_2 = x, -x; ϕ_3 = x, x, -x, -x; ϕ_4 = x, -x; ϕ_5 = x, x, -x, -x; ϕ_6 = x, x, -x, -x; ϕ_7 = x; ϕ_8 = 4x,4(-x); ϕ_9 = x; ϕ_{10}(receiver) = x, -x, -x, x. The

sensitivity enhancement scheme of Kay (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) is employed, *i.e.*, the sign of G5 is inverted in concert with a 180° shift of ϕ_9. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_2 and ϕ_7, respectively, according to States-TPPI (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with $\phi_1 = $ -x is collected. The sum and the difference of the two resulting data sets generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0029]   Figure 3D illustrates the experimental scheme for the 3D HNNCAHA experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, $\phi_8 = $ y. The scaling factor κ for ^1H chemical shift evolution during t_1 is set to 1.0. The 90° pulse lengths were: 5.8 μs for ^1H and 21.6 μs for $^{13}C^\alpha$, and 38 μs for ^{15}N, where the 90° pulse width for $^{13}C^\alpha$ is adjusted to generate a null of excitation in the center of the CO chemical shift range. The selective 90° ^1H pulse used to flip back the water magnetization is applied for the 1.8 ms with the SEDUCE-1 profile. WALTZ16 is employed to decouple ^1H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple of ^{15}N (r.f. = 1.78 kHz) during acquisition. SEDUCE is used for decoupling of ^{13}CO (max. r.f. = 3.0 kHz). WURST-2 is used for simultaneous band selective decoupling of ^{13}CO and $^{13}C^\beta$ during τ_4 and the ^1H and ^{13}C chemical shift evolution during t_1. 3.0 kHz sweeps at 176 ppm and 30 ppm, respectively, are used for decoupling of ^{13}CO and $^{13}C^\beta$ (except for Ser, Thr, Ala). A sweep of 600 Hz is used at 14 ppm to decouple $^{13}C^\beta$ of Ala. The ^1H r.f. carrier is placed at the position of the solvent line at 4.78 ppm for the first three ^1H pulses and the first WALTZ period, then switched to 0 ppm during the first delay $\tau_4/2$, and subsequently switched back to the water line at 4.78 ppm during t_1^c. The $^{13}C^\alpha$ and ^{15}N carriers are set to 56.1 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 μs, 8

G/cm); G2 (500 µs, 4 G/cm); G3 (1 ms, 30 G/cm); G4 (150 µs, 25 G/cm); G5 (1.25 ms, 30 G/cm); G6 (500 µs, 8G/cm); G7 (125 µs, 29.57 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 µs duration is inserted between a PFG pulse and an r.f. pulse. The delays have the following

5　values: τ_1 = 4.6 ms, τ_2 = 5.5 ms, τ_3 = 24 ms, τ_4 = 2.0 ms, τ_5 = 500 µs. ^{13}C-frequency labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.065 ms, t_1^b (0) = 49 µs, t_1^c (0) = 984 µs, Δt_1^a = 65 µs, Δt_1^b = 49 µs, Δt_1^c = -16 µs. Hence, the fractional increase of the semi constant-time period with t_1 equals to λ = 1 + Δt_1^c / Δt_1^a = 0.76. Note that the acquisition starts with the second complex

10　point in t_1, while the first one is obtained by linear prediction. This ensures that a zero first-order phase correction is achieved along ω_1. Phase cycling: ϕ_1 = x, -x; ϕ_2 = x, x, -x, -x; ϕ_3 = x, -x, -x, x; ϕ_4 =x; ϕ_5 = 4(x), 4(-x); ϕ_6 = x; ϕ_7(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby

15　incorporated by reference in its entirety) is employed, i.e., the sign of G5 is inverted in concert with a 180° shift of ϕ_6. Quadrature detection in t_1(^{13}C) and t_2(^{15}N) is accomplished by altering the phases ϕ_2 and ϕ_4 according to States-TPPI (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety).

20　**[0030]**　Figure 3E illustrates the experimental scheme for the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for the ^{1}H

25　chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.9 µs for ^{1}H, 15.4 µs for ^{13}C, and 38.2 µs for ^{15}N. The 90° and 180° pulse lengths of ^{13}C$^{\alpha/\beta}$ were adjusted to 47.4 µs and 42.4 µs, respectively, to minimize perturbation of ^{13}CO spins. A 200 µs 180° pulse with SEDUCE profile is used to selectively invert ^{13}CO magnetization prior to the start of t_1. The 90° and 180°

30　pulses employed for excitation of ^{13}CO and subsequent magnetization transfer back to ^{13}C$^{\alpha}$ are of rectangular shape and 52 µs and 103 µs duration, respectively.

The length of the spin-lock purge pulses SL_x and SL_y are 2.5 ms and 1 ms, respectively. WALTZ16 is employed to decouple ^1H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers, and for decoupling of ^{15}N (r.f. = 1.78 kHz) during acquisition. GARP is used for decoupling of $^{13}C^\alpha$ (r.f. =

5   2.5 kHz). The ^1H r.f. carrier is placed at the position of the solvent line at 0 ppm before the start of the first semi constant time ^1H evolution period and then switched to the water line at 4.78 ppm after the second 90° ^1H pulse. Initially, the ^{13}C and ^{15}N r.f. carriers are set to 43 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 = G2 (100

10   µs, 15 G/cm); G3 (2 ms, 25 G/cm); G4 (100 µs, 10 G/cm); G5 (1 ms, 27 G/cm); G6 (3 ms, 30 G/cm); G7 (1.3 ms, 20 G/cm); G8 (130 µs, 14 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 µs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 800 µs, τ_2 = 2.8 ms, τ_3 = 3.6 ms, τ_4 = 6.5 ms, τ_5 = 1.8 ms, τ_6 = 1 ms, τ_7 = 2.8 ms, τ_8 = 3.6 ms.

15   ^1H-frequency labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 µs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 µs, Δt_1^b = 19.3 µs, Δt_1^c = -14 µs. Hence, the fractional increase of the semi constant-time period with t_1 equals to λ = 1 + Δt_1^c / Δt_1^a = 0.58. Phase cycling: ϕ_1 = x; ϕ_x = x, -x; ϕ_3 = x, -x, x, -x; ϕ_4 = x; ϕ_5(receiver) = x, -x. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is

20   accomplished by altering the phases ϕ_2 and ϕ_4, respectively, according to States-TPPI (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 = -x is collected. The sum and the difference of the two resulting data sets

25   generate subspectra II and I, respectively, containing the central peaks and peak pairs (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996), which is hereby incorporated by reference in its entirety).

[0031]   Figure 3F illustrates the experimental scheme for the 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN experiment. Rectangular 90° and 180° pulses are indicated by thin

30   and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In

order to accomplish sine modulation, $\phi_1 = y$. The scaling factor κ for the ^1H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.9 μs for ^1H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power, and ^{13}C decoupling during $t_1(^1H)$ is achieved

5   using a $(90_x\text{-}180_y\text{-}90_x)$ composite pulse. Subsequently, the 90° and 180° pulse lengths of $^{13}C^{\alpha/\beta}$ are adjusted to 49 μs and 43.8 μs to minimize perturbation of ^{13}CO spins. SEDUCE 180° pulses of 200μs pulse length are used to decouple ^{13}CO. WALTZ16 is employed to decouple ^1H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers, as well as to decouple ^{15}N (r.f. =

10   1.78 kHz). The ^1H carrier is placed at the position of the solvent line at 0 ppm during the first semi constant time ^1H evolution period, and then switched to the water line 4.78 ppm after the second 90° ^1H pulse. The ^{13}C and ^{15}N r.f. carriers are set to 43 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (500 μs, 8 G/cm);

15   G3 (250 μs, 15 G/cm); G4 (1 ms, 11 G/cm); G5 (500 μs, 20 G/cm); G6(500 μs, 4 G/cm); G7 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. The delays are: $\tau_1 = 800$ μs, $\tau_2 = 2.8$ ms, $\tau_3 = 3.3$ ms, $\tau_4 = 7.2$ ms, $\tau_5 = 24$ ms, $\tau_6 = 5.4$ ms, $\tau_7 = 4.8$ ms, $\tau_8 = 1$ ms. ^1H-frequency labeling is achieved in a semi constant-time fashion with $t_1^a (0) = 1.7$ ms, $t_1^b (0) = 1$ μs, $t_1^c (0) = 1.701$ ms, $\Delta t_1^a = 33.3$ μs,

20     $\Delta t_1^b = 19.3$ μs, $\Delta t_1^c = -14$ μs. Hence, the fractional increase of the semi constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c / \Delta t_1^a = 0.58$. Phase cycling: $\phi_1 = x$; $\phi_2 = x$; $\phi_3 = x,\text{-}x$; $\phi_4 = x, x, \text{-}x, \text{-}x$; $\phi_5 = x$; $\phi_6(\text{receiver}) = x, \text{-}x$. The sensitivity enhancement scheme of Kay (Cavanagh et al., <u>Protein NMR Spectroscopy,</u> Academic Press, San Diego, (1996), which is hereby incorporated by reference in

25   its entirety) is employed, $i.e.$, the sign of G5 is inverted in concert with a 180° shift of ϕ_5. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_2 and ϕ_3, respectively, according to States-TPPI. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with $\phi_1 = \text{-}x$ is collected. The sum and the difference of the two resulting data sets

30   generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0032] Figure 3G illustrates the experimental scheme for the 3D HNN<u>CO,CA</u>> experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the . pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied

5   along x. In order to accomplish sine modulation, ϕ_3 = y. The scaling factor κ for $^{13}C^\alpha$ chemical shift evolution during t_2 is set to 0.5. The high power 90° pulse lengths were: 5.8 μs for ^1H and 38.5 μs for ^{15}N. The 90° and 180° pulse lengths of $^{13}C^\alpha$ were adjusted 54 μs and 48.8 μs to minimize perturbation of ^{13}CO spins. The length of the 90° pulses applied on ^{13}CO are 102 μs, and they possess the

10   shape of a sinc center lobe. The corresponding 180° pulses are applied with same power and shape. The selective ^1H 90° pulse used for flip-back of water magnetization is applied for 1.8 ms with the SEDUCE-1 profile. WALTZ16 is employed to decouple ^1H (r.f. field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during acquisition (r.f. = 1.78

15   kHz). The SEDUCE sequence is used for decoupling of $^{13}C^\alpha$ during ^{15}N evolution period (r.f. = 0.9 kHz). The $^{13}C^\alpha$ and ^{15}N r.f. carriers are set to 176.5 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 μs, 30 G/cm); G2 (500 μs, 5 G/cm); G3 (2 ms, 13 G/cm); G4 (750 μs, 20 G/cm); G5 (200 μs, 5 G/cm); G6 (100 μs, 12 G/cm); G7

20   (1.25 ms, 30 G/cm); G8 (300 μs, 5 G/cm); G9 (200 μs, 10 G/cm); G10 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 4.6 ms, τ_2 = 5.5 ms, τ_3 = τ_4 = 28 ms, τ_5 = 1 ms. Phase cycling: ϕ_1 = x, x, -x, -x; ϕ_2 = x, -x; ϕ_3 = x; ϕ_4 = x; ϕ_5 = 4(x), 4(-x); ϕ_6 = x; ϕ_7(receiver) = x, -x, -x, x. The

25   sensitivity enhancement scheme of Kay (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) is employed, i.e., the sign of G5 is inverted in concert with a 180° shift of ϕ_6. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_2 and ϕ_4, respectively, according to States-TPPI (Cavanagh

30   et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). To shift the apparent $^{13}C^\alpha$ carrier position to 82.65 ppm, i.e., downfield to all $^{13}C^\alpha$ resonances, ϕ_3 is incremented in

60° steps according to TPPI. Note, that the acquisition was started with the ninth complex point and the first eight complex points along $\omega_1(^{13}CO)$ were obtained by linear prediction. This ensures that a zero first-order phase correction is achieved along ω_1 (Szyperski et al., J. Magn. Reson., B 108: 197–203 (1995), which is hereby incorporated by reference in its entirety).

[0033] Figure 3H illustrates the experimental scheme for the 3D HCCH-COSY experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for ^{1}H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^{1}H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. The lengths of the ^{1}H spin-lock purge pulses are: first SL_x, 2.8 ms; second SL_x, 1.7 ms; SL_y: 4.9 ms. SEDUCE is used for decoupling of ^{13}CO during t_1 and t_2 (r.f. field strength = 1 kHz). WURST is used for decoupling of ^{13}C during acquisition. The ^{1}H carrier is placed at the position of the solvent line at 0 ppm before the start of the first semi constant time ^{1}H evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^{1}H pulse. The ^{13}C and ^{15}N r.f. carriers are set to 38 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 μs, 6 G/cm); G2 (500 μs, 7 G/cm); G3 (100 μs, 12 G/cm); G4 (100 μs, 12.5 G/cm); G5 (2 ms, 9 G/cm);G6 (500 μs, 5 G/cm); G7 (1.5 ms, 8 G/cm); G8 (400 μs, 6 G/cm). All gradients are applied along z-axis and are of rectangular shape. All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 1.6 ms, τ_2 = 850 μs, τ_3 = 2.65 ms, τ_4 = 3.5 ms, τ_5 = 7 ms, τ_6 = 1.6 ms, τ_7 = 3.2 ms. Phase cycling: ϕ_1 = x; ϕ_2 = x, -x; ϕ_3 = x, -x; ϕ_4 = x; ϕ_5(receiver) = x, -x. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{13}C)$ is accomplished by altering the phases ϕ_2 and ϕ_3, respectively, according to States-TPPI (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 = -x is collected.

The sum and the difference of the two resulting data sets generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0034] Figure 3I illustrates the experimental scheme for the 3D HCCH-TOCSY experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, ϕ_1 = y. The scaling factor κ for ^{1}H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^{1}H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. ^{13}C decoupling during t_1(^{1}H) is achieved using a (90_x-180_y-90_x) composite pulse. The lengths of the ^{1}H spin-lock purge pulses are: first SL_x, 5.7 ms; second SL_x, 0.9 ms; SL_y, 4.3 ms. SEDUCE is used for decoupling of ^{13}CO during t_1 and t_2 (r.f. field strength = 1 kHz), and GARP is employed for decoupling of ^{13}C during acquisition (r.f. = 2.5 kHz). The ^{1}H r.f. carrier is placed at the position of the solvent line at 0 ppm before the start of the first semi constant time ^{1}H evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^{1}H pulse. The ^{13}C$^\alpha$ and ^{15}N r.f. carriers are set to 38 ppm and 120.9 ppm, respectively. The length of ^{13}C spin-lock purge pulses denoted SL_x are of 2 ms duration. ^{13}C isotropic mixing is accomplished using the DIPSI-2 scheme (r.f. = 8.5 kHz). The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (100 μs, 16 G/cm); G2 (2 ms, 15 G/cm); G3 (300 μs, 8 G/cm); G4 (500 μs, 30 G/cm); G5 (100 μs, 16 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 850 μs, τ_2 = 3.2 ms. ^{1}H-frequency labeling in t_1 is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 μs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 μs, Δt_1^b = 19.3 μs, Δt_1^c = - 14 μs. ^{13}C-frequency labeling in t_2 is achieved in a semi constant-time fashion with t_2^a (0) = 1120 μs, t_2^b (0) = 62.5 μs, t_2^c (0) = 995 μs, Δt_2^a = 160 μs, Δt_2^b = 125 μs, Δt_2^c = -35 μs. These delays ensure that a 90° first-order phase correction is obtained along ω_2(^{13}C). The fractional increases of the semi constant-time period in t_1 equals to λ = 1 + Δt_2^c / Δt_2^a = 0.58, and in t_2 equals to λ = 1 + Δt_2^c / Δt_2^a = 0.78. Phase cycling: ϕ_1 = x; ϕ_2 = x, -x; ϕ_3 = x; ϕ_4 = 2(x), 2(-x); ϕ_5(receiver) = x,-

x. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{13}C)$ is accomplished by altering the phases ϕ_2 and ϕ_3, respectively, according to States-TPPI (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with $\phi_1 = -x$ is collected. The sum and the difference of the two resulting data sets generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0035] Figure 3J illustrates the experimental scheme for the 2D HBCB(CGCD)HD experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, $\phi_1 = y$. The scaling factor κ for ^1H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^1H and 15.4 μs for ^{13}C. The first 180° pulse on ^{13}C prior to $t_1(^{13}C)$ is applied at high power. Subsequently, the 90° pulse lengths of $^{13}C^\beta$ is adjusted to 66 μs. The 180° $^{13}C^\beta$ and $^{13}C^{aro}$ pulses are of gaussian-3 shape and 375 μs duration. WALTZ16 is used for decoupling of ^1H (r.f. field strength = 4.5 kHz) during the magnetization transfer from $^{13}C^\alpha$ to $^{13}C^{aro}$, and GARP is employed to decouple $^{13}C^{aro}$ (r.f. = 2.5 kHz) during acquisition. The ^1H r.f. carrier is placed at 0 ppm before the start of the semi constant time ^1H evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^1H pulse. The ^{13}C r.f. carrier is set to 38 ppm during $\omega_1(^{13}C^\beta)$ and then switched to 131 ppm before the first 90° pulse on $^{13}C^{aro}$ (pulse labeled with ϕ_4). The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 μs, 2 G/cm); G2 (1 ms, 22 G/cm); G3 (2 ms, 10 G/cm); G4 (1 ms, 5 G/cm); G5 (500 μs, 4 G/cm); G6 (1 ms, -14 G/cm); G7 (500 μs, -2G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: $\tau_1 = 1.8$ ms, $\tau_2 = 8.8$ ms, $\tau_3 = 71$ μs, $\tau_4 = 5.4$ ms, $\tau_5 = 4.2$ ms, $\tau_6 = 710$ μs, $\tau_7 = 2.5$ ms. ^1H-frequency labeling is achieved in a semi constant-time fashion with $t_1^a(0) = 1.7$ ms, $t_1^b(0) = 1$ μs, $t_1^c(0) = 1.701$ ms, $\Delta t_1^a = 33.3$ μs, $\Delta t_1^b = 19.3$ μs, $\Delta t_1^c = -14$ μs. Hence, the fractional increase of the semi

constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c / \Delta t_1^a = 0.58$. Phase cycling: ϕ_1 = x; ϕ_2 = x; ϕ_3 = x, y, -x, -y; ϕ_4 = 4(x), 4(-x); ϕ_5 (receiver) = x, -x, x, -x, -x, x, -x, x. Quadrature detection in $t_1(^{13}C)$ is accomplished by altering the phases ϕ_2 respectively, according to States-TPPI. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 = -x is collected. The sum and the difference of the two resulting data sets generate subspectra II and I, respectively, containing the central peaks and peak pairs.

[0036] Figure 3K illustrates the experimental scheme for the 2D ^1H-TOCSY-relayed-<u>HC</u>H-COSY experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (r.f.) phase is marked, the pulse is applied along x. In order to accomplish sine modulation, ϕ_6 = y. The high-power 90° pulse lengths were: 5.9 μs for ^1H and 15.4 μs for ^{13}C. The ^1H r.f. carrier is placed at the position of the solvent line at 4.78 ppm, and the ^{13}C carrier is set to 131 ppm. GARP is used for ^{13}C decoupling during acquisition (r.f. field strength = 2.5 kHz), and ^1H isotropic mixing is accomplished using the DIPSI-2 scheme (r.f. = 16 kHz). The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, -10 G/cm); G2 (500 μs, 6 G/cm); G3 (500 μs, 7.5 G/cm); G4 (1 ms, 22 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an r.f. pulse. The delays are: τ_1 = 3.0 ms, τ_2 = 15.38 ms. Phase cycling: ϕ_1 = x, -x; ϕ_2 = x, x, y, y, -x, -x, -y, -y; ϕ_3 = 4(x),4(y), 4(-x), 4(-y); ϕ_4 = x, x, -x, -x; ϕ_5(receiver) = x, -x, x, -x, -x, x, -x, x. Quadrature detection in $t_1(^{13}C)$ is accomplished by altering the phase ϕ_1 according to States-TPPI.

[0037] Figure 4 illustrates the polypeptide chemical shifts correlated by the various spectra constituting the TR NMR experiments identified for efficient HTP resonance assignment of proteins. The nuclei for which the chemical shifts are obtained from a given experiment are boxed and labeled accordingly.

[0038] Figure 5 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^N)]$-strips taken from 3D <u>HACA</u>(CO)NHN spectrum obtained for Z-domain protein with a total measurement time of 6.7 hours. The 3D NMR data were acquired with 40(t_1)

× 28(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C^{\alpha}; {}^1H^{\alpha}) = 6.5$ ms, $t_{2max}(^{15}N) = 20.8$ ms, and $t_{3max}(^1H^N) = 73.2$ ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peak" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peak shows opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N chemical shift (113.8 ppm) of Asp 50 along $\omega_2(^{15}N)$ and centered about the corresponding amide proton shifts detected along $\omega_3(^1H^N)$. ^1H, ^{13}C, and ^{15}N chemical shifts are in ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS).

[0039] Figure 6 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^N)]$-strips taken from 3D HNNCAHA spectrum obtained for Z-domain protein with a total measurement time of 10.7 hours. The 3D NMR data were acquired with 64(t_1) × 28(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C^{\alpha}; {}^1H^{\alpha}) = 12.1$ ms, $t_{2max}(^{15}N) = 20.8$ ms, and $t_{3max}(^1H^N) = 73.2$ ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from incomplete INEPT transfer of the ^{13}C magnetization. The strip labeled "central peak" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peak shows opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and

strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N chemical shift (117.4 ppm) of Leu 35 along $\omega_2(^{15}$N) and centered about the corresponding amide proton shifts detected along $\omega_3(^1$H$^N)$. ^{1}H, ^{13}C, and ^{15}N chemical shifts are in ppm relative to DSS.

[0040]   Figure 7 shows a composite plot of $[\omega_1(^{13}$C/^{1}H),$\omega_3(^1$H$^N)]$-strips taken from 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN spectrum obtained for Z-domain protein with a total measurement time of 12.7 hours. The 3D NMR data were acquired with 76(t_1) × 28(t_2) × 512(t_3) complex points and $t_{1max}(^{13}$C$^{\alpha/\beta}$; ^{1}H$^{\alpha/\beta}$) = 6.25 ms, $t_{2max}(^{15}$N) = 20.8 ms, and $t_{3max}(^1$H$^N)$ = 73.2 ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N chemical shift (117.4 ppm) of Leu 35 along $\omega_2(^{15}$N) and centered about the corresponding amide proton shifts detected along $\omega_3(^1$H$^N)$. ^{1}H, ^{13}C, and ^{15}N chemical shifts are in ppm relative to DSS.

[0041]   Figure 8 shows a composite plot of $[\omega_1(^{13}$C/^{1}H),$\omega_3(^1$H$^N)]$-strips taken from 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN spectrum obtained for Z-domain protein with a total measurement time of 11.4 hours. The 3D NMR data were acquired with 68(t_1) × 28(t_2) × 512(t_3) complex points and $t_{1max}(^{13}$C$^{\alpha/\beta}$; ^{1}H$^{\alpha/\beta}$) = 5.6 ms, $t_{2max}(^{15}$N) = 20.8 ms, and $t_{3max}(^1$H$^N)$ = 73.2 ms. Data acquisition and processing were performed as

shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine

5   modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the

10   3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N chemical shift (117.4 ppm) of Leu 35 along $\omega_2(^{15}N)$ and centered about the corresponding amide proton shifts detected along $\omega_3(^1H^N)$. ^1H, ^{13}C, and ^{15}N

15   chemical shifts are in ppm relative to DSS.

[0042]   Figure 9 shows a composite plot of $[\omega_1(^{13}C'/^{13}C^\alpha),\omega_3(^1H^N)]$-strips taken from 3D HNN<CO,CA> spectrum obtained for Z-domain protein with a total measurement time of 9.4 hours. The 3D NMR data were acquired with $56(t_1)$ $\times 28(t_2) \times 512(t_3)$ complex points and $t_{1max}(^{13}C'; {}^{13}C^\alpha) = 6.9$ ms, $t_{2max}(^{15}N) = 20.8$

20   ms, and $t_{3max}(^1H^N) = 73.2$ ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peak" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine

25   modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the

30   3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N

chemical shift (170.0 ppm) of Phe 43 along $\omega_2(^{15}N)$ and centered about the corresponding amide proton shifts detected along $\omega_3(^1H^N)$. ^1H, ^{13}C, and ^{15}N chemical shifts are in ppm relative to DSS.

[0043]   Figure 10 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^N)]$-strips taken from 3D HC(C-TOCSY-CO)NHN spectrum obtained for Z-domain protein with a total measurement time of 12.7 hours. The 3D NMR data were acquired with 76(t_1) × 28(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C; ^1H)$ = 6.3 ms, $t_{2max}(^{15}N)$ = 20.8 ms, and $t_{3max}(^1H^N)$ = 73.2 ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{15}N chemical shift (118.8 ppm) of Phe 18 along $\omega_2(^{15}N)$ and centered about the corresponding amide proton shifts detected along $\omega_3(^1H^N)$. ^1H, ^{13}C, and ^{15}N chemical shifts are in ppm relative to DSS.

[0044]   Figure 11 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^\beta)]$-strips taken from 3D HCCH-COSY spectrum obtained for Z-domain protein with a total measurement time of 10.8 hours. The 3D NMR data were acquired with 90(t_1) × 20(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C; ^1H)$ = 7.4 ms, $t_{2max}(^{13}C)$ = 6.1 ms, and $t_{3max}(^1H)$ = 73.2 ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine

modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D

5 NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference between the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the $^{13}C^{\beta}$ chemical shift (37.8 ppm) of Asn 19 along $\omega_2(^{13}C)$ and centered about the

10 corresponding proton ($^1H^{\beta}$) shifts detected along $\omega_3(^1H)$. ^1H and ^{13}C chemical shifts are in ppm relative to DSS.

[0045]   Figure 12 shows a composite plot of [$\omega_1(^{13}C/^1H),\omega_3(^1H^{\beta})$]-strips taken from 3D H̲C̲CH-TOCSY spectrum obtained for Z-domain protein with a total measurement time of 11.0 hours. The 3D NMR data were acquired with

15 80(t_1) × 20(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C; ^1H)$ = 6.6 ms, $t_{2max}(^{13}C)$ = 6.1 ms, and $t_{3max}(^1H)$ = 73.2 ms. Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the

20 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D

25 NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference of the cosine-modulated 3D NMR spectrum between the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the $^{13}C^{\beta}$ chemical shift (37.8 ppm) of Asn 36 along $\omega_2(^{13}C)$ and centered about the

30 corresponding proton ($^1H^{\beta}$) shifts detected along $\omega_3(^1H)$. ^1H and ^{13}C chemical shifts are in ppm relative to DSS.

[0046] Figure 13 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^\delta)]$-strips taken from 2D HBCB(CGCD)HD spectrum obtained for Z-domain protein with a total measurement time of 7.8 hours. The 2D NMR data were acquired with 76(t_1) × 512(t_2) complex points and $t_{1max}(^{13}C;\ ^1H) = 6.3$ ms and $t_{2max}(^1H) = 73.2$ ms.

5 Data acquisition and processing were performed as shown in Figure 1. The central peaks were acquired from steady state ^{13}C magnetization. The strip labeled "central peak" was taken from the 2D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 2D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the

10 2D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 2D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 2D NMR spectrum and the sine-modulated 2D NMR spectrum, and strip (D) from the 2D NMR spectrum

15 comprising the edited, phase-sensitive peak generated from the difference of the cosine-modulated 2D NMR spectrum between the sine-modulated 2D NMR spectrum. All strips have been centered about the aromatic proton ($^1H^\delta$) shifts of Phe 18 detected along $\omega_2(^1H)$. ^1H and ^{13}C chemical shifts are in ppm relative to DSS.

20 **[0047]** Figure 14 shows a composite plot of $[\omega_1(^{13}C/^1H),\omega_3(^1H^\alpha)]$-strips taken from 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA spectrum obtained for ubiquitin protein with a total measurement time of 12.7 hours. The 3D NMR data were acquired with 76(t_1) × 32(t_2) × 512(t_3) complex points and $t_{1max}(^{13}C^{\alpha/\beta};\ ^1H^{\alpha/\beta}) = 6.3$ ms, $t_{2max}(^{13}C') = 17.2$ ms, and $t_{3max}(^1H^\alpha) = 72.8$ ms. Data acquisition and processing were performed as

25 shown in Figure 1. The central peaks were acquired from the steady state ^{13}C magnetization. The strip labeled "central peaks" was taken from the 3D NMR spectrum comprising the central peaks. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak

30 pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D

NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference of the cosine-modulated 3D NMR spectrum between the sine-modulated 3D NMR spectrum. All strips have been taken, respectively, at the ^{13}C' chemical shift (175.2 ppm) of Pro 19 along $\omega_2(^{13}$C') and centered about the corresponding proton shifts detected along $\omega_3(^1$H$^\alpha)$. ^{1}H and ^{13}C chemical shifts are in ppm relative to DSS.

[0048] Figure 15 shows a composite plot of $[\omega_1(^{13}$C/^{1}H),$\omega_3(^1$H)]-strips taken from 2D H-TOCSY-HCH-COSY spectrum obtained for ubiquitin protein with a total measurement time of 1.4 hours. The 2D NMR data were acquired with $100(t_1) \times 512(t_2)$ complex points and $t_{1max}(^{13}$C; ^{1}H) = 9.9 ms and $t_{2max}(^1$H) = 72.8 ms. Data acquisition and processing were performed as shown in Figure 1. No central peaks were acquired. The strip labeled (A) was taken from the 3D NMR spectrum comprising in-phase peak pairs arising from cosine modulation; strip (B) from the 3D NMR spectrum comprising anti-phase peak pairs arising from sine modulation (the boxed peaks show opposite peak intensity compared to the others); strip (C) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the sum of the cosine-modulated 3D NMR spectrum and the sine-modulated 3D NMR spectrum, and strip (D) from the 3D NMR spectrum comprising the edited, phase-sensitive peak generated from the difference of the cosine-modulated 3D NMR spectrum between the sine-modulated 3D NMR spectrum. All strips have been centered about the aromatic proton shifts detected along $\omega_2(^1$H). ^{1}H and ^{13}C chemical shifts are in ppm relative to DSS.

DETAILED DESCRIPTION OF THE INVENTION

[0049] The present invention discloses eleven RD TR NMR experiments where the chemical shift values encoded in a peak pair of an NMR spectrum are detected in a phase sensitive manner. The present invention also discloses different combinations of those phase-sensitively detected RD NMR experiments which allow one to obtain sequential backbone chemical shift assignments for

determining the secondary structure of a protein molecule and nearly complete assignments of chemical shift values for a protein molecule including aliphatic and aromatic sidechain spin systems.

[0050]   The basic acquisition principle for recording edited, phase sensitive RD NMR spectra is illustrated in Figure 1 (Brutscher et al., J. Magn. Reson., B 109:238-242 (1995), which is hereby incorporated by reference in its entirety). The joint evolution of two chemical shifts, Ω_x and Ω_y, can result in four different time domain NMR data sets, (1), (2), (3), and (4), which are generated from all possible combinations of sine modulation and cosine modulation with either of the two chemical shifts (shown on the left-hand side of the equations for data sets (1) – (4)). According to the addition theorems of trigonometric functions, these products of sine and cosine functions are equal to sums of sine and cosine functions of sums and differences of the two chemical shifts (shown on the right-hand side of the equations for data sets (1) – (4)). To obtain an absorptive NMR signal, two out of the four data sets need to be considered as "real" and "imaginary" parts for cosine-modulated RD NMR and sine-modulated RD NMR. Data sets (1) and (2) correspond to real part and imaginary part, respectively, of a cosine-modulated RD NMR experiment, in which Ω_y is detected in a phase sensitive manner and the evolution of the chemical shift Ω_x effects a cosine modulation (see (A) in Figure 1). Data sets (3) and (4) correspond to real part and imaginary part, respectively, of a sine-modulated RD NMR experiment, in which Ω_y is detected in a phase sensitive manner and the evolution of Ω_x effects a sine modulation, further enabling the phase sensitive detection of Ω_x (see (B) in Figure 1). Thus, the phase-sensitively detected sine-modulated RD NMR spectrum contains all information about Ω_x and Ω_y for unambiguous chemical shift assignment. Appropriate linear combination of real and imaginary parts of the cosine-modulated and the sine-modulated RD NMR experiments yields the edited, phase sensitive RD NMR spectra, which also contain all information about Ω_x and Ω_y (see (C) and (D) in Figure 1).

[0051]   Figure 2 provides a survey of (i) the names, (ii) the magnetization transfer pathways and (iii) the peak patterns observed in the projected dimension of specific embodiments of the eleven RD NMR experiments disclosed by the

present invention. For simplicity, Figure 2 shows the in-phase peak pattern from the cosine-modulated RD NMR experiments. When acquiring the sine-modulated congeners, anti-phase peak pairs are observed instead (see (B) in Figure 1). The group comprising the first three experiments are designed to yield "sequential"

5   connectivities via one-bond scalar couplings: 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN (Figure 2A), 3D HACA(CO)NHN (Figure 2B), and 3D HC(C-TOCSY-CO)NHN (Figure 2C). The following three experiments provide "intraresidual" connectivities via one-bond scalar couplings: 3D HNN$\underline{CAHA}$ (Figure 2D), 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA (Figure 2E), and 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN (Figure 2F). 3D HNN<$\underline{CO,CA}$> (Figure 2G) offers

10   both intraresidual $^{1}H^{N}$–$^{13}C^{\alpha}$ and sequential $^{1}H^{N}$–$^{13}C'$ connectivities. Although 3D HNN$\underline{CAHA}$ (Figure 2D), 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN (Figure 2F) and 3D HNN<$\underline{CO,CA}$> (Figure 2G) also provide sequential connectivities via two-bond $^{13}C^{\alpha}_{i-1}$–$^{15}N_i$ scalar couplings, those are usually smaller than the one-bond couplings (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby

15   incorporated by reference in its entirety), and obtaining complete backbone resonance assignments critically depends on experiments designed to provide sequential connectivities via one-bond couplings (Figures 2D-F). 3D HCCH-COSY (Figure 2H) and 3D HCCH-TOCSY (Figure 2I) allow one to obtain assignments for the "aliphatic" side chain spin systems, while 2D

20   HBCB(CDCG)HD (Figure 2J) and 2D ^{1}H-TOCSY-relayed HCH-COSY (Figure 2K) provide the corresponding information for the "aromatic" spin systems.

[0052]   The RD NMR experiments are grouped accordingly in Table 1, which lists for each experiment (i) the nuclei for which the chemical shifts are measured, (ii) if and how the central peaks are acquired and (iii) additional notable

25   technical features. State-of-the art implementations (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996); Kay, J. Am. Chem. Soc., 115:2055–2057 (1993); Grzesiek et al., J. Magn. Reson., 99:201–207 (1992); Montelione et al., J. Am. Chem. Soc., 114:10974–10975 (1992); Boucher et al., J. Biomol. NMR, 2:631–637 (1992); Yamazaki et al., J. Am. Chem. Soc.,

30   115:11054–11055 (1993); Zerbe et al., J. Biomol. NMR, 7:99–106 (1996); Grzesiek et al., J. Biomol. NMR, 3:185–204 (1993), which are hereby incorporated by reference in their entirety) making use of pulsed field z-gradients

for coherence selection and/or rejection, and sensitivity enhancement (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) were chosen, which allow executing these experiments with a single transient per acquired free induction

5   decay (FID). Semi (Grzesiek et al., J. Biomol. NMR, 3:185–204 (1993), which is hereby incorporated by reference in its entirety) constant-time (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) chemical shift frequency-labeling modules were used throughout in the indirect dimensions in order to minimize

10   losses arising from transverse nuclear spin relaxation. Figures 3A-K provide comprehensive descriptions of the RD NMR r.f. pulse sequences used in the eleven RD NMR experiments.

Table 1. Phase-Sensitively Detected Reduced Dimensionality NMR Experiments

Experiment (see Figure 2)	Nuclei for which the chemical shifts are correlated[a,b]	Acquisition of central peaks[c]
3D spectra for sequential backbone connectivities:		
(A) $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN	$^1H^\beta_{i-1}$, $^{13}C^\beta_{i-1}$, $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, $^{15}N_i$, $^1H^N_i$	^{13}C
(B) $\underline{HACA}$(CO)NHN	$^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, $^{15}N_i$, $^1H^N_i$	^{13}C
(C) $\underline{HC}$(C-TOCSY- CO)NHN	$^1H^{ali}_{i-1}$, $^{13}C^{ali}_{i-1}$, $^{15}N_i$, $^1H^N_i$	^{13}C
3D spectra for intraresidual backbone connectivities:		
(D) HNN$\underline{CAHA}$[b,d]	$^1H^\alpha_i$, $^{13}C^\alpha_i$, $^{15}N_i$, $^1H^N_i$	INEPT
(E) $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA	$^1H^\beta_i$, $^{13}C^\beta_i$, $^1H^\alpha_i$, $^{13}C^\alpha_i$, $^{13}C=O_i$	^{13}C
(F) $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NH	$^1H^\beta_i$, $^{13}C^\beta_i$, $^1H^\alpha_i$, $^{13}C^\alpha_i$, $^{15}N_i$, $^1H^N_i$	^{13}C
3D spectrum for intra- and sequential backbone connectivities:		
(G) HNN<$\underline{CO,CA}$>[b]	$^{13}C=O_{i-1}$, $^{13}C^\alpha_i$, $^{15}N_i$, $^1H^N_i$	INEPT
3D spectra for assignment of aliphatic resonances:[e]		
(H) $\underline{HC}$CH-COSY	^1H_m, $^{13}C_m$, ^1H_n, $^{13}C_n$	^{13}C
(I) $\underline{HC}$CH-TOCSY	^1H_m, $^{13}C_m$, ^1H_n, $^{13}C_n$, ^1H_p, $^{13}C_p$	^{13}C
2D spectra for assignment of aromatic resonances:[e]		
(J) $\underline{HB}$CB(CGCD)HD	$^1H^\beta$, $^{13}C^\beta$, $^1H^\delta$	^{13}C
(K) ^{1}H-TOCSY-$\underline{HC}$H-COSY	^1H_m, $^{13}C_m$, ^1H_n,	none[f]

[a] i-1, i: numbers of two sequentially located amino acid residues.

[b] Sequential connectivities via two-bond $^{13}C^\alpha_{i-1}$-$^{15}N_i$ scalar couplings are not considered in this table.

[c] approach-1 (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996), which is hereby incorporated by reference in its entirety): use of incomplete INEPT (rows labeled with "INEPT"); approach-2 (Szyperski et al., J. Am. Chem. Soc., 118:8146-8147 (1996), which is hereby incorporated by reference in its entirety): use of ^{13}C steady state magnetization (rows labeled with "^{13}C").

[d] adiabatic $^{13}C^\beta$-decoupling (Kupce et al., J. Magn. Reson., A 115:273–277 (1995); Matsuo et al., J. Magn. Reson. B 113:190–194 (1996), which are hereby incorporated by reference in their entirety) is employed during delays with transverse $^{13}C^\alpha$ magnetization.

[e] m, n, p: atom numbers in neighboring CH, CH_2 or CH_3 groups.

[f] acquisition of central peaks is prevented by the use of spin-lock purge pulses (flanking the total correlation relay) to obtain pure phases.

The 3D HA,CA,(CO),N,HN experiment

[0053]   The present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) HA,CA,(CO),N,HN nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having two consecutive amino acid residues, i-1 and i: (1) an α-proton of amino acid residue i-1, $^1H^\alpha_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^\alpha_{i-1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, where the chemical shift values of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ of amino acid residue i-1 are connected to the chemical shift evolutions of $^{15}N_i$ and $^1H^N_i$ of amino acid residue i, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^\alpha_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^\alpha)$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^1H^N_i$ in a direct time domain dimension, $t_3(^1H^N)$, and (2) to sine modulate the $^{13}C^\alpha_{i-1}$ chemical shift evolution in $t_1(^{13}C^\alpha)$ with the chemical shift evolution of $^1H^\alpha_{i-1}$. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with an anti-phase peak pair derived from the sine modulating, where (1) the chemical shift values of $^{15}N_i$ and $^1H^N_i$ are measured in two frequency domain dimensions, $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$, respectively, and (2) the chemical shift values of $^1H^\alpha_{i-1}$ and $^{13}C^\alpha_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^\alpha)$, by the frequency difference between the two peaks forming the anti-phase peak pair and the frequency at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of $^1H^\alpha_{i-1}$ in a phase sensitive manner.

[0054]   In addition, the method of conducting a RD 3D HA,CA,(CO),N,HN NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the

chemical shift values of $^{13}C^{\alpha}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in $t_1(^{13}C^{\alpha})$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^{1}H^{N}_i$ in $t_3(^{1}H^{N})$ and (2) to cosine modulate the $^{13}C^{\alpha}_{i-1}$ chemical shift evolution in $t_1(^{13}C^{\alpha})$ with the chemical shift evolution of $^{1}H^{\alpha}_{i-1}$ for the additional NMR signals. Then, the NMR signals

5   and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with an in-phase peak pair derived from the cosine modulating, a sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-modulated 3D NMR spectrum, and a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum

10   from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0055]   In addition, the method of conducting a RD 3D

15   HA,CA,(CO),N,HN NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^{\alpha}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in $t_1(^{13}C^{\alpha})$ and $t_2(^{15}N)$ and the chemical shift value of $^{1}H^{N}_i$ in $t_3(^{1}H^{N})$, and (2) to avoid sine modulating the $^{13}C^{\alpha}_{i-1}$ chemical shift evolution in $t_1(^{13}C^{\alpha})$ with the chemical shift

20   evolution of $^{1}H^{\alpha}_{i-1}$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR spectrum with an additional peak located centrally between two peaks forming the primary peak pair which measures the chemical shift value of $^{13}C^{\alpha}_{i-1}$ along $\omega_1(^{13}C^{\alpha})$. That additional peak can be derived from $^{13}C^{\alpha}$ nuclear spin polarization. One specific

25   embodiment (3D HACA(CO)NHN) of this method is illustrated in Figure 2B, where the applying radiofrequency pulses effects a nuclear spin polarization transfer where a radiofrequency pulse is used to create transverse $^{1}H^{\alpha}_{i-1}$ magnetization, which is transferred to $^{13}C^{\alpha}_{i-1}$, to $^{15}N_i$, and to $^{1}H^{N}_i$, to generate the NMR signal. Another specific embodiment of this method involves applying

30   radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3B to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown

in Figure 3B, where phase ϕ_1 of the first ^{1}H pulse is altered by 180° to generate a second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted whereby the NMR signals are processed to generate a first NMR subspectrum derived from the subtracting which contains the primary peak pair and a second NMR subspectrum derived from the adding which contains the additional peak located centrally between the two peaks forming the primary peak pair.

[0056]   In an alternate embodiment, the RD 3D HA,CA,(CO),N,HN NMR experiment can be modified to a RD 2D HA,CA,(CO,N),HN NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of ^{15}N$_i$ does not occur. Then, the NMR signals are processed to generate a two dimensional (2D) NMR spectrum with a peak pair where (1) the chemical shift value of ^{1}H^N_i is measured in a frequency domain dimension, $\omega_2(^1$H$^N)$, and (2) the chemical shift values of ^{1}H$^\alpha_{i-1}$ and ^{13}C$^\alpha_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}$C$^\alpha)$, by the frequency difference between the two peaks forming the peak pair and the frequency at the center of the two peaks, respectively.

[0057]   In an alternate embodiment, the RD 3D HA,CA,(CO),N,HN NMR experiment can be modified to a RD 4D HA,CA,CO,N,HN NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of a polypeptide backbone carbonyl carbon of amino acid residue i-1, ^{13}C'$_{i-1}$, occurs under conditions effective to generate NMR signals encoding the chemical shift value of ^{13}C'$_{i-1}$ in a phase sensitive manner in an indirect time domain dimension, $t_4(^{13}$C'). Then, the NMR signals are processed to generate a four dimensional (4D) NMR spectrum with a peak pair where (1) the chemical shift values of ^{15}N$_i$, ^{1}H^N_i and ^{13}C'$_{i-1}$ are measured in three frequency domain dimensions, $\omega_2(^{15}$N), $\omega_3(^1$H$^N)$, and $\omega_4(^{13}$C'), respectively, and (2) the chemical shift values of ^{1}H$^\alpha_{i-1}$ and ^{13}C$^\alpha_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}$C$^\alpha)$, by the frequency difference between the two peaks forming the peak pair and the frequency at the center of the two peaks, respectively.

The 3D <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN experiment

[0058] The present invention also relates to a method of conducting a reduced dimensionality three-dimensional (3D) <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having two consecutive amino acid residues, i-1 and i: (1) aliphatic protons of amino acid residue i-1, $^1H^{ali}_{i-1}$; (2) aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}_{i-1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, where the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ of amino acid residue i-1 are connected to the chemical shift evolutions of $^{15}N_i$ and $^1H^N_i$ of amino acid residue i, under conditions effective (1) to generate a NMR signal encoding the chemical shifts of $^{13}C^{ali}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^{ali})$ and $t_2(^{15}N)$, respectively, and the chemical shift of $^1H^N_i$ in a direct time domain dimension, $t_3(^1H^N)$, and (2) to sine modulate the chemical shift evolutions of $^{13}C^{ali}_{i-1}$ in $t_1(^{13}C^{ali})$ with the chemical shift evolutions of $^1H^{ali}_{i-1}$. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{15}N_i$ and $^1H^N_i$ are measured in two frequency domain dimensions, $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$, respectively, and (2) the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{ali})$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of $^1H^{ali}_{i-1}$ in a phase sensitive manner.

[0059] In addition, the method of conducting a RD 3D <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical

shift values of $^{13}C^{ali}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in $t_1(^{13}C^{ali})$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^1H^N_i$ in $t_3(^1H^N)$ and (2) to cosine modulate the chemical shift evolutions of $^{13}C^{ali}_{i-1}$ in $t_1(^{13}C^{ali})$ with the chemical shift evolutions of $^1H^{ali}_{i-1}$ for the additional NMR signals. Then, the NMR signals

5 and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with in-phase peak pairs derived from the cosine modulating, a sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-modulated 3D NMR spectrum, and a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum

10 from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0060] In addition, the method of conducting a RD 3D H,C,(C-TOCSY-

15 CO),N,HN NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^{ali}_{i-1}$ and $^{15}N_i$ in a phase sensitive manner in $t_1(^{13}C^{ali})$ and $t_2(^{15}N)$ and the chemical shift value of $^1H^N_i$ in $t_3(^1H^N)$, and (2) to avoid sine modulating the chemical shift evolutions of $^{13}C^{ali}_{i-1}$ in $t_1(^{13}C^{ali})$ with the chemical shift

20 evolution of $^1H^{\alpha}_{i-1}$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR spectrum with additional peaks located centrally between two peaks forming the peak pairs which measure the chemical shift values of $^{13}C^{ali}_{i-1}$ along $\omega_1(^{13}C^{ali})$. Those additional peaks can be derived from $^{13}C^{ali}$ nuclear spin polarization. One specific

25 embodiment (3D HC-(C-TOCSY-CO)NHN) of this method is illustrated in Figure 2C, where the applying radiofrequency pulses effects a nuclear spin polarization transfer, where a radiofrequency pulse is used to create transverse $^1H^{ali}_{i-1}$ magnetization, and $^1H^{ali}_{i-1}$ magnetization is transferred to $^{13}C^{ali}_{i-1}$, to $^{13}C^{\alpha}_{i-1}$, to $^{13}C'_{i-1}$, to $^{15}N_i$, and to $^1H^N_i$, where the NMR signal is detected. Another specific

30 embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3C to generate a first NMR signal, and (2) applying a second set of

radiofrequency pulses according to the scheme shown in Figure 3C, where phase ϕ_1 of the first ^1H pulse is altered by $180°$ to generate a second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are processed to generate a first

5   NMR subspectrum derived from the subtracting which contains the peak pairs, and a second NMR subspectrum derived from the adding which contains the additional peaks located centrally between the two peaks forming the peak pairs.

[0061]   In an alternate embodiment, the RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment can be modified to a RD 2D $\underline{H},\underline{C},$(C-TOCSY-

10   CO,N),HN NMR experiment, which involves applying radiofrequency pulses so that the chemical shift evolution of $^{15}N_i$ does not occur. Then, the NMR signals are processed to generate a two dimensional (2D) NMR spectrum with peak pairs where (1) the chemical shift value of $^1H^N_i$ is measured in a frequency domain dimension, $\omega_2(^1H^N)$, and (2) the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ are

15   measured in a frequency domain dimension, $\omega_1(^{13}C^{ali})$, by the frequency differences between the two peaks forming the peak pairs and the frequencies at the center of the two peaks, respectively.

[0062]   In an alternate embodiment, the RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment can be modified to a RD 4D $\underline{H},\underline{C},$(C-

20   TOCSY),CO,N,HN NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, occurs under conditions effective to generate NMR signals encoding the chemical shift value of $^{13}C'_{i-1}$ in a phase sensitive manner in an indirect time domain dimension, $t_4(^{13}C')$. Then, the NMR

25   signals are processed to generate a four dimensional (4D) NMR spectrum with variant peak pairs where (1) the chemical shift values of $^{15}N_i$, $^1H^N_i$ and $^{13}C'_{i-1}$ are measured in three frequency domain dimensions, $\omega_2(^{15}N)$, $\omega_3(^1H^N)$, and $\omega_4(^{13}C')$, respectively, and (2) the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{ali})$, by the frequency differences between

30   the two peaks forming the variant peak pairs and the frequencies at the center of the two peaks, respectively.

The 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},CO,HA$ experiment

[0063] Another aspect of the present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},CO,HA$ nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having an amino acid residue, i: (1) a β-proton of amino acid residue i, $^1H^\beta_i$; (2) a β-carbon of amino acid residue i, $^{13}C^\beta_i$; (3) an α-proton of amino acid residue i, $^1H^\alpha_i$; (4) an α-carbon of amino acid residue i, $^{13}C^\alpha_i$; and (5) a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$, where the chemical shift values of $^1H^\alpha_i/^{13}C^\alpha_i$ and $^1H^\beta_i/^{13}C^\beta_i$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^\alpha_i$, $^1H^\beta_i$, $^{13}C^\alpha_i$, and $^{13}C^\beta_i$ are connected to the chemical shift evolution of $^{13}C'_i$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^\alpha_i$, $^{13}C^\beta_i$ and $^{13}C'_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{13}C')$, respectively, and the chemical shift value of $^1H^\alpha_i$ in a direct time domain dimension, $t_3(^1H^\alpha)$, and (2) to sine modulate the chemical shift evolutions of $^{13}C^\alpha_i$ and $^{13}C^\beta_i$ in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^1H^\alpha_i$ and $^1H^\beta_i$, respectively. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{13}C'_i$ and $^1H^\alpha_i$ are measured in two frequency domain dimensions, $\omega_2(^{13}C')$ and $\omega_3(^1H^\alpha)$, respectively, and (2) (i) the chemical shift values of $^1H^\alpha_i$ and $^1H^\beta_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs, and (ii) the chemical shift values of $^{13}C^\alpha_i$ and $^{13}C^\beta_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the anti-phase peak pairs, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift values of $^1H^\alpha_i$ and $^1H^\beta_i$ in a phase sensitive manner.

[0064] In addition, the method of conducting a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^{\alpha}_{i}$, $^{13}C^{\beta}_{i}$ and $^{13}C'_{i}$ in a phase sensitive manner in $t_1(^{13}C^{\alpha/\beta})$ and

5 $t_2(^{13}C')$, respectively, and the chemical shift value of $^{1}H^{\alpha}_{i}$ in $t_3(^{1}H^{\alpha})$ and (2) to cosine modulate the $^{13}C^{\alpha}_{i}$ and $^{13}C^{\beta}_{i}$ chemical shift evolutions in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^{1}H^{\alpha}_{i}$ and $^{1}H^{\beta}_{i}$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with in-phase peak pairs derived from the

10 cosine modulating, a sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-modulated 3D NMR spectrum, and a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables

15 placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0065] In addition, the method of conducting a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift

20 values of $^{13}C^{\alpha}_{i}$, $^{13}C^{\beta}_{i}$ and $^{15}N_{i}$ in a phase sensitive manner in $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{15}N)$ and the chemical shift value of $^{1}H^{\alpha}_{i}$ in $t_3(^{1}H^{\alpha})$, and (2) to avoid cosine modulating the chemical shift evolutions of $^{13}C^{\alpha}_{i}$ and $^{13}C^{\beta}_{i}$ in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^{1}H^{\alpha}_{i}$ and $^{1}H^{\beta}_{i}$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR

25 spectrum with additional peaks located centrally between two peaks forming the peak pairs which measure the chemical shift values of $^{13}C^{\alpha}_{i}$ and $^{13}C^{\beta}_{i}$ along $\omega_1(^{13}C^{\alpha/\beta})$. Those additional peaks can be derived from $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ nuclear spin polarization. One specific embodiment (3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA) of this method is illustrated in Figure 2E, where the applying radiofrequency pulses effects a

30 nuclear spin polarization transfer, where a radiofrequency pulse is used to create transverse $^{1}H^{\alpha}_{i}$ and $^{1}H^{\beta}_{i}$ magnetization, and $^{1}H^{\alpha}_{i}$ and $^{1}H^{\beta}_{i}$ polarization is

transferred to $^{13}C^{\alpha}_i$ and $^{13}C^{\beta}_i$, to $^{13}C'_i$, and back to $^{1}H^{\alpha}_i$, where the NMR signal is detected. Another specific embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3E to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown in Figure 3E, where phase ϕ_1 of the first ^{1}H pulse is altered by $180°$ to generate a second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are processed to generate a first NMR subspectrum derived from the subtracting which contains the peak pairs, and a second NMR subspectrum derived from the adding which contains the additional peaks located centrally between the two peaks forming the peak pairs.

[0066] In an alternate embodiment, the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},CO,HA$ NMR experiment can be modified to a RD 2D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},(CO),HA$ NMR experiment, which involves applying radiofrequency pulses so that the chemical shift evolution of $^{13}C'_i$ does not occur. Then, the NMR signals are processed to generate a two dimensional (2D) NMR spectrum with peak pairs where (1) the chemical shift value of $^{1}H^{\alpha}_i$ is measured in a frequency domain dimension, $\omega_2(^{1}H^{\alpha})$, and (2) (i) the chemical shift values of $^{1}H^{\alpha}_i$ and $^{1}H^{\beta}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequency differences between two peaks forming the peak pairs, respectively, and (ii) the chemical shift values of $^{13}C^{\alpha}_i$, and $^{13}C^{\beta}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the peak pairs.

The 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ experiment

[0067] A further aspect of the present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule having an amino acid residue, i: (1) a β-proton of amino acid residue i, $^{1}H^{\beta}_i$; (2) a β-carbon of amino acid residue i, $^{13}C^{\beta}_i$; (3) an α-proton of amino acid residue i, $^{1}H^{\alpha}_i$; (4) an α-carbon

of amino acid residue i, $^{13}C^{\alpha}{}_i$; (5) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (6) a polypeptide backbone amide proton of amino acid residue i, $^1H^N{}_i$, where the chemical shift values of $^1H^{\alpha}{}_i/^{13}C^{\alpha}{}_i$ and $^1H^{\beta}{}_i/^{13}C^{\beta}{}_i$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase

5   sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^{\alpha}{}_i$, $^1H^{\beta}{}_i$, $^{13}C^{\alpha}{}_i$, and $^{13}C^{\beta}{}_i$ are connected to the chemical shift evolutions of $^{15}N_i$ and $^1H^N{}_i$, under conditions effective (1) to generate NMR signals encoding the chemical shift

10   values of $^{13}C^{\alpha}{}_i$, $^{13}C^{\beta}{}_i$ and $^{15}N_i$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^1H^N{}_i$ in a direct time domain dimension, $t_3(^1H^N)$, and (2) to sine modulate the chemical shift evolutions of $^{13}C^{\alpha}{}_i$ and $^{13}C^{\beta}{}_i$ in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^1H^{\alpha}{}_i$ and $^1H^{\beta}{}_i$, respectively. Then, the NMR signals are processed to

15   generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{15}N_i$ and $^1H^N{}_i$ are measured in two frequency domain dimensions, $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$, respectively, and (2) (i) the chemical shift values of $^1H^{\alpha}{}_i$ and $^1H^{\beta}{}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequency differences between

20   each of the two peaks forming each of the anti-phase peak pairs, and (ii) the chemical shift values of $^{13}C^{\alpha}{}_i$ and $^{13}C^{\beta}{}_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the anti-phase peak pairs, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift values of $^1H^{\alpha}{}_i$ and $^1H^{\beta}{}_i$ in a phase sensitive manner.

25   **[0068]**   In addition, the method of conducting a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^{\alpha}{}_i$, $^{13}C^{\beta}{}_i$ and $^{15}N_i$ in a phase sensitive manner in $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{15}N)$, respectively, and the chemical shift value of $^1H^N{}_i$ in $t_3(^1H^N)$ and (2) to cosine

30   modulate the $^{13}C^{\alpha}{}_i$ and $^{13}C^{\beta}{}_i$ chemical shift evolutions in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^1H^{\alpha}{}_i$ and $^1H^{\beta}{}_i$ for the additional NMR signals. Then,

the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with in-phase peak pairs derived from the cosine modulating, a sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-modulated 3D NMR spectrum, and

5 a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

10 **[0069]** In addition, the method of conducting a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^{\alpha}{}_{i}$, $^{13}C^{\beta}{}_{i}$ and $^{15}N_{i}$ in a phase sensitive manner in $t_1(^{13}C^{\alpha/\beta})$ and $t_2(^{15}N)$ and the chemical shift value of $^{1}H^{N}{}_{i}$ in $t_3(^{1}H^{N})$, and (2) to avoid cosine modulating

15 the chemical shift evolutions of $^{13}C^{\alpha}{}_{i}$ and $^{13}C^{\beta}{}_{i}$ in $t_1(^{13}C^{\alpha/\beta})$ with the chemical shift evolutions of $^{1}H^{\alpha}{}_{i}$ and $^{1}H^{\beta}{}_{i}$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR spectrum with additional peaks located centrally between two peaks forming the peak pairs which measure the chemical shift values of $^{13}C^{\alpha}{}_{i}$ and $^{13}C^{\beta}{}_{i}$ along

20 $\omega_1(^{13}C^{\alpha/\beta})$. Those additional peaks can be derived from $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ nuclear spin polarization. One specific embodiment (3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}NHN$) of this method is illustrated in Figure 2F, where the applying radiofrequency pulses effects a nuclear spin polarization transfer where a radiofrequency pulse is used to create transverse $^{1}H^{\alpha}{}_{i}$ and $^{1}H^{\beta}{}_{i}$ magnetization, and $^{1}H^{\alpha}{}_{i}$ and $^{1}H^{\beta}{}_{i}$ magnetization is

25 transferred to $^{13}C^{\alpha}{}_{i}$ and $^{13}C^{\beta}{}_{i}$, to $^{15}N_{i}$, and to $^{1}H^{N}{}_{i}$, where the NMR signal is detected. Another specific embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3F to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown

30 in Figure 3F, where phase ϕ_1 of the first ^{1}H pulse is altered by 180° to generate a second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are

processed to generate a first NMR subspectrum derived from the subtracting which contains the peak pairs, and a second NMR subspectrum derived from the adding which contains the additional peaks located centrally between the two peaks forming the peak pairs.

[0070]   In an alternate embodiment, the RD 3D $\underline{H}^{\alpha/\beta}, \underline{C}^{\alpha/\beta}, N, HN$ NMR experiment can be modified to a RD 2D $\underline{H}^{\alpha/\beta}, \underline{C}^{\alpha/\beta}, (N), HN$ NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of $^{15}N_i$ does not occur. Then, the NMR signals are processed to generate a two dimensional (2D) NMR spectrum with peak pairs where (1) the chemical shift value of $^1H^N_i$ is measured in a frequency domain dimension, $\omega_2(^1H^N)$, and (2) (i) the chemical shift values of $^1H^\alpha_i$ and $^1H^\beta_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequency differences between the two peaks forming the peak pairs, and (ii) the chemical shift values of $^{13}C^\alpha_i$, and $^{13}C^\beta_i$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\alpha/\beta})$, by the frequencies at the center of the two peaks forming the peak pairs.

The 3D $\underline{H}, \underline{C}, C, H$-COSY experiment

[0071]   The present invention also relates to a method of conducting a reduced dimensionality three-dimensional (3D) $\underline{H}, \underline{C}, C, H$-COSY nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for $^1H^m$, $^{13}C^m$, $^1H^n$, and $^{13}C^n$ of a protein molecule where m and n indicate atom numbers of two CH, CH$_2$ or CH$_3$ groups that are linked by a single covalent carbon-carbon bond in an amino acid residue, where the chemical shift values of $^1H^m$ and $^{13}C^m$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effects a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^m$ and $^{13}C^m$ are connected to the chemical shift evolutions of $^1H^n$ and $^{13}C^n$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$, respectively, and the chemical shift value of $^1H^n$ in a direct time domain dimension, $t_3(^1H^n)$, and (2) to sine modulate the chemical shift

evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of ^1H_m. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{13}C^n$ and $^1H^n$ are measured in two frequency domain dimensions,

5    $\omega_2(^{13}C^n)$ and $\omega_3(^1H^n)$, respectively, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value

10   of ^1H_m in a phase sensitive manner.

[0072]   In addition, the method of conducting a RD 3D H,C,C,H-COSY NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$,

15   respectively, and the chemical shift value of $^1H^n$ in $t_3(^1H^n)$, and (2) to cosine modulate the $^{13}C^m$ chemical shift evolution in $t_1(^{13}C^m)$ with the chemical shift evolution of ^1H_m for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with in-phase peak pairs derived from the cosine modulating, a

20   sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-modulated 3D NMR spectrum, and a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables placement of the two

25   peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0073]   In addition, the method of conducting a RD 3D H,C,C,H-COSY NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift

30   values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$ and the chemical shift value of $^1H^n$ in $t_3(^1H)$, and (2) to avoid cosine modulating the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of

$^1H^m$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR spectrum with additional peaks located centrally between two peaks forming the peak pairs which measure the chemical shift value of $^{13}C^m$ along $\omega_1(^{13}C^m)$. Those additional peaks can be

5   derived from $^{13}C^m$ nuclear spin polarization. One specific embodiment (3D HCCH-COSY) of this method is illustrated in Figure 2H, where the applying radiofrequency pulses effects a nuclear spin polarization transfer, where a radiofrequency pulse is used to create transverse $^1H^m$ magnetization, and $^1H^m$ magnetization is transferred to $^{13}C^m$, to $^{13}C^n$, and to $^1H^n$, where the NMR signal is

10   detected. Another specific embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3H to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown in Figure 3H, where phase ϕ_1 of the first ^1H pulse is altered by 180° to generate a

15   second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are processed to generate a first NMR subspectrum derived from the subtracting which contains the peak pairs, and a second NMR subspectrum derived from the adding which contains the additional peaks located centrally between the two

20   peaks forming the peak pairs.

[0074]   In an alternate embodiment, the RD 3D H,C,C,H-COSY NMR experiment can be modified to a RD 2D H,C,(C),H-COSY NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of $^{13}C^n$ does not occur. Then, the NMR signals are processed to

25   generate a two dimensional (2D) NMR spectrum with peak pairs where (1) the chemical shift value of $^1H^n$ is measured in a frequency domain dimension, $\omega_2(^1H^n)$, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between the two peaks forming the peak pairs and the frequencies at the center of the two

30   peaks, respectively.

The 3D H,C,C,H-TOCSY experiment

[0075]   Another aspect of the present invention relates to a method of conducting a reduced dimensionality three-dimensional (3D) H,C,C,H-TOCSY nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for $^1H^m$, $^{13}C^m$, $^1H^n$, and $^{13}C^n$ of a protein molecule where m and n indicate atom numbers of two CH, CH_2 or CH_3 groups that may or may not be directly linked by a single covalent carbon-carbon bond in an amino acid residue, where the chemical shift values of $^1H^m$ and $^{13}C^m$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^m$ and $^{13}C^m$ are connected to the chemical shift evolutions of $^1H^n$ and $^{13}C^n$, under conditions effective (1) to generate NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in two indirect time domain dimensions, $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$, and the chemical shift value of $^1H^n$ in a direct time domain dimension, $t_3(^1H^n)$, and (2) to sine modulate the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of $^1H^m$. Then, the NMR signals are processed to generate a sine-modulated 3D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift values of $^{13}C^n$ and $^1H^n$ are measured in two frequency domain dimensions, $\omega_2(^{13}C^n)$ and $\omega_3(^1H^n)$, respectively, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 3D NMR spectrum enables detection of the chemical shift value of ^1H_m in a phase sensitive manner.

[0076]   In addition, the method of conducting a RD 3D H,C,C,H-TOCSY NMR can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$, respectively, and the chemical shift value of $^1H^n$ in $t_3(^1H^n)$ and (2) to cosine modulate the $^{13}C^m$ chemical shift evolution in $t_1(^{13}C^m)$ with the chemical shift evolution of ^1H_m for the

additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 3D NMR spectrum with in-phase peak pairs derived from the cosine modulating, a sum 3D NMR spectrum generated by adding the sine-modulated 3D NMR spectrum and the cosine-

5   modulated 3D NMR spectrum, and a difference 3D NMR spectrum generated by subtracting the cosine-modulated 3D NMR spectrum from the sine-modulated 3D NMR spectrum. The combined use of the sum 3D NMR spectrum and the difference 3D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the

10   two peaks forming the peak pairs.

[0077]   In addition, the method of conducting a RD 3D H̲,C̲,C,H-TOCSY NMR can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift values of $^{13}C^m$ and $^{13}C^n$ in a phase sensitive manner in $t_1(^{13}C^m)$ and $t_2(^{13}C^n)$ and the chemical shift

15   value of $^1H^n$ in $t_3(^1H^n)$, and (2) to avoid cosine modulating the chemical shift evolution of $^{13}C^m$ in $t_1(^{13}C^m)$ with the chemical shift evolution of $^1H^m$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 3D NMR spectrum with additional peaks located centrally between two peaks forming the peak pairs which measure the chemical

20   shift value of $^{13}C^m$ along $\omega_1(^{13}C^m)$. Those additional peaks can be derived from $^{13}C^m$ nuclear spin polarization. One specific embodiment (3D H̲C̲CH-TOCSY) of this method is illustrated in Figure 2I, where the applying radiofrequency pulses effects a nuclear spin polarization transfer where a radiofrequency pulse is used to create transverse $^1H^m$ magnetization, and $^1H^m$ magnetization is transferred to $^{13}C^m$,

25   to $^{13}C^n$, and to $^1H^n$, where the NMR signal is detected. Another specific embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3I to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown in Figure 3I, where phase

30   ϕ_1 of the first ^1H pulse is altered by 180° to generate a second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are processed to generate a first

NMR subspectrum derived from the subtracting which contains the peak pairs, and a second NMR subspectrum derived from the adding which contains the additional peaks located centrally between the two peaks forming the peak pairs.

[0078] In an alternate embodiment, the RD 3D H,C,C,H-TOCSY NMR experiment can be modified to a RD 2D H,C,(C),H-TOCSY NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of $^{13}C^n$ does not occur. Then, the NMR signals are processed to generate a two dimensional (2D) NMR spectrum with peak pairs where (1) the chemical shift value of $^1H^n$ is measured in a frequency domain dimension, $\omega_2(^1H^n)$, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between the two peaks forming the peak pairs and the frequencies at the center of the two peaks, respectively.

The 2D HB,CB,(CG,CD),HD experiment

[0079] A further aspect of the present invention relates to a method of conducting a reduced dimensionality two-dimensional (2D) HB,CB,(CG,CD),HD nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for the following nuclei of a protein molecule: (1) a β-proton of an amino acid residue with an aromatic side chain, $^1H^\beta$; (2) a β-carbon of an amino acid residue with an aromatic side chain, $^{13}C^\beta$; and (3) a δ-proton of an amino acid residue with an aromatic side chain, $^1H^\delta$, where the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ which are encoded in a peak pair of a 2D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^1H^\beta$ and $^{13}C^\beta$ are connected to the chemical shift evolution of $^1H^\delta$, under conditions effective (1) to generate NMR signals encoding the chemical shift value of $^{13}C^\beta$ in a phase sensitive manner in an indirect time domain dimension, $t_1(^{13}C^\beta)$, and the chemical shift value of $^1H^\delta$ in a direct time domain dimension, $t_2(^1H^\delta)$, and (2) to sine modulate the chemical shift evolution of $^{13}C^\beta$ in $t_1(^{13}C^\beta)$ with the chemical shift

evolution of $^1H^\beta$. Then, the NMR signals are processed to generate a sine-modulated 2D NMR spectrum with an anti-phase peak pair derived from the sine modulating where (1) the chemical shift value of $^1H^\delta$ is measured in a frequency domain dimension, $\omega_2(^1H^\delta)$, and (2) the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ are measured in a frequency domain dimension, $\omega_1(^{13}C^\beta)$, by the frequency difference between the two peaks forming the anti-phase peak pair and the frequency at the center of the two peaks, respectively, where the sine-modulated 2D NMR spectrum enables detection of the chemical shift value of $^1H^\beta$ in a phase sensitive manner.

[0080]   In addition, the method of conducting a RD 2D HB,CB,(CG,CD),HD NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift value of $^{13}C^\beta$ in a phase sensitive manner in $t_1(^{13}C^\beta)$ and the chemical shift value of $^1H^\delta$ in $t_2(^1H^\delta)$ and (2) to cosine modulate the $^{13}C^\beta$ chemical shift evolution in $t_1(^{13}C^\beta)$ with the chemical shift evolution of $^1H^\beta$ for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 2D NMR spectrum with an in-phase peak pair derived from the cosine modulating, a sum 2D NMR spectrum generated by adding the sine-modulated 2D NMR spectrum and the cosine-modulated 2D NMR spectrum, and a difference 2D NMR spectrum generated by subtracting the cosine-modulated 2D NMR spectrum from the sine-modulated 2D NMR spectrum. The combined use of the sum 2D NMR spectrum and the difference 2D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0081]   In addition, the method of conducting a RD 2D HB,CB,(CG,CD),HD NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift value of $^{13}C^\beta$ in a phase sensitive manner in $t_1(^{13}C^\beta)$ and the chemical shift value of $^1H^\delta$ in $t_2(^1H^\delta)$, and (2) to avoid cosine modulating the chemical shift evolution of $^{13}C^\beta$ in $t_1(^{13}C^\beta)$ with the chemical shift evolution of $^1H^\beta$

for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to generate a 2D NMR spectrum with an additional peak located centrally between the two peaks forming the peak pair which measure the chemical shift value of $^{13}C^\beta$ along $\omega_1(^{13}C)$. That additional peak can be derived

5   from $^{13}C^\beta$ nuclear spin polarization. One specific embodiment (2D HBCB(CGCD)HD) of this method is illustrated in Figure 2J, where the applying radiofrequency pulses effects a nuclear spin polarization transfer where a radiofrequency pulse is used to create transverse $^1H^\beta$ magnetization, and $^1H^\beta$ magnetization is transferred to $^{13}C^\beta$, to $^{13}C^\delta$, and to $^1H^\delta$, where the NMR signal is

10   detected. Another specific embodiment of this method involves applying radiofrequency pulses by (1) applying a first set of radiofrequency pulses according to the scheme shown in Figure 3J to generate a first NMR signal, and (2) applying a second set of radiofrequency pulses according to the scheme shown in Figure 3J, where phase ϕ_1 of the first ^1H pulse is altered by 180° to generate a

15   second NMR signal. Then, prior to the processing, the first NMR signal and the second NMR signal are added and subtracted, whereby the NMR signals are processed to generate a first NMR subspectrum derived from the subtracting which contains the peak pair, and a second NMR subspectrum derived from the adding which contains the additional peak located centrally between the two peaks

20   forming the peak pair.

[0082]   In an alternate embodiment, the RD 2D HB,CB,(CG,CD),HD NMR experiment can be modified to a RD 3D HB,CB,(CG),CD,HD NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of a δ-carbon of an amino acid residue with an aromatic side chain,

25   $^{13}C^\delta$ occurs under conditions effective to generate NMR signals encoding the chemical shift value of $^{13}C^\delta$ in a phase sensitive manner in an indirect time domain dimension, $t_3(^{13}C^\delta)$. Then, the NMR signals are processed to generate a three dimensional (3D) NMR spectrum with a peak pair where (1) the chemical shift values of $^1H^\delta$ and $^{13}C^\delta$ are measured in two frequency domain dimensions, $\omega_2(^1H^\delta)$

30   and $\omega_3(^{13}C^\delta)$, respectively, and (2) the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ are measured in a frequency domain dimension, $\omega_1(^{13}C^\beta)$, by the frequency difference

between the two peaks forming the peak pair and the frequency at the center of the two peaks, respectively.

[0083] In an alternate embodiment, the RD 2D HB,CB,(CG,CD),HD NMR experiment can be modified to a RD 3D HB,CB,CG,(CD),HD NMR experiment which involves applying radiofrequency pulses so that the chemical shift evolution of a γ-carbon of an amino acid residue with an aromatic side chain, $^{13}C^{\gamma}$ occurs under conditions effective to generate NMR signals encoding the chemical shift value of $^{13}C^{\gamma}$ in a phase sensitive manner in an indirect time domain dimension, $t_3(^{13}C^{\gamma})$, and said processing the NMR signals generates a three dimensional (3D) NMR spectrum with a peak pair wherein (1) the chemical shift values of $^{1}H^{\delta}$ and $^{13}C^{\gamma}$ are measured in two frequency domain dimensions, $\omega_2(^{1}H^{\delta})$ and $\omega_3(^{13}C^{\gamma})$, respectively, and (2) the chemical shift values of $^{1}H^{\beta}$ and $^{13}C^{\beta}$ are measured in a frequency domain dimension, $\omega_1(^{13}C^{\beta})$, by the frequency difference between the two peaks forming said peak pair and the frequency at the center of the two peaks, respectively.

The 2D H,C,H-COSY experiment

[0084] The present invention also relates to a method of conducting a reduced dimensionality two-dimensional (2D) H,C,H-COSY nuclear magnetic resonance (NMR) experiment by measuring the chemical shift values for $^{1}H^{m}$, $^{13}C^{m}$, and $^{1}H^{n}$ of a protein molecule where m and n indicate atom numbers of two CH, CH_2 or CH_3 groups in an amino acid residue, where the chemical shift values of $^{1}H^{m}$ and $^{13}C^{m}$ which are encoded in a peak pair of a 2D NMR spectrum are detected in a phase sensitive manner. The method involves providing a protein sample and applying radiofrequency pulses to the protein sample which effect a nuclear spin polarization transfer where the chemical shift evolutions of $^{1}H^{m}$ and $^{13}C^{m}$ are connected to the chemical shift evolution of $^{1}H^{n}$, under conditions effective (1) to generate NMR signals encoding the chemical shift value of $^{13}C^{m}$ in a phase sensitive manner in an indirect time domain dimension, $t_1(^{13}C^{m})$, and the chemical shift value of $^{1}H^{n}$ in a direct time domain dimension, $t_2(^{1}H^{n})$, and (2) to sine modulate the chemical shift evolution of $^{13}C^{m}$ in $t_1(^{13}C^{m})$ with the chemical shift evolution of $^{1}H^{m}$. Then, the NMR signals are processed to generate a sine-

modulated 2D NMR spectrum with anti-phase peak pairs derived from the sine modulating where (1) the chemical shift value of $^1H^n$ is measured in a frequency domain dimension, $\omega_2(^1H^n)$, and (2) the chemical shift values of $^1H^m$ and $^{13}C^m$ are measured in a frequency domain dimension, $\omega_1(^{13}C^m)$, by the frequency differences between each of the two peaks forming each of the anti-phase peak pairs and the frequencies at the center of the two peaks, respectively, where the sine-modulated 2D NMR spectrum enables detection of the chemical shift value of ^1H_m in a phase sensitive manner.

[0085] In addition, the method of conducting a RD 2D H,C,H-COSY NMR experiment can involve applying radiofrequency pulses under conditions effective (1) to generate additional NMR signals encoding the chemical shift value of $^{13}C^m$ in a phase sensitive manner in $t_1(^{13}C^m)$ and the chemical shift value of $^1H^n$ in $t_2(^1H^n)$ and (2) to cosine modulate the $^{13}C^m$ chemical shift evolution in $t_1(^{13}C^m)$ with the chemical shift evolution of ^1H_m for the additional NMR signals. Then, the NMR signals and the additional NMR signals are processed to further generate a cosine-modulated 2D NMR spectrum with in-phase peak pairs derived from the cosine modulating, a sum 2D NMR spectrum generated by adding the sine-modulated 2D NMR spectrum and the cosine-modulated 2D NMR spectrum, and a difference 2D NMR spectrum generated by subtracting the cosine-modulated 2D NMR spectrum from the sine-modulated 2D NMR spectrum. The combined use of the sum 2D NMR spectrum and the difference 2D NMR spectrum enables placement of the two peaks forming the peak pairs into separate spectra, thereby allowing phase-sensitive editing of the two peaks forming the peak pairs.

[0086] One specific embodiment (2D ^1H-TOCSY-HCH-COSY) of this method is illustrated in Figure 2K, where the applying radiofrequency pulses effects a nuclear spin polarization transfer where a radiofrequency pulse is used to create transverse $^1H^m$ magnetization, and $^1H^m$ polarization is transferred to $^{13}C^m$, to $^1H^m$, and to $^1H^n$, where the NMR signal is detected. Although the specific embodiment illustrated in Figure 2K shows this method applied to an amino acid residue with an aromatic side chain, this method also applies to amino acid residues with aliphatic side chains. Another specific embodiment of this method

involves applying radiofrequency pulses according to the scheme shown in Figure 3K.

[0087] Figure 4 outlines which chemical shifts are correlated in the various NMR experiments described above.

Combinations of RD NMR Experiments

[0088] Accordingly, a suite of multidimensional RD NMR experiments enables one to devise strategies for RD NMR-based HTP resonance assignment of proteins.

[0089] Thus, another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^1\text{H}^\alpha$, an α-carbon, $^{13}\text{C}^\alpha$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1\text{H}^{N,}$ of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1\text{H}^\alpha$ and $^{13}\text{C}^\alpha$ which are encoded in a peak pair of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D HA,CA,(CO),N,HN NMR experiment to measure and connect chemical shift values of the α-proton of amino acid residue i-1, $^1\text{H}^\alpha_{i\text{-}1}$, the α-carbon of amino acid residue i-1, $^{13}\text{C}^\alpha_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}\text{N}_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1\text{H}^N_i$ and (2) a RD 3D HNNCAHA NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i, $^1\text{H}^\alpha_i$, the α-carbon of amino acid residue i, $^{13}\text{C}^\alpha_i$, $^{15}\text{N}_i$, and $^1\text{H}^N_i$. Then, sequential assignments of the chemical shift values of $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, ^{15}N, and $^1\text{H}^N$ are obtained by (i) matching the chemical shift values of $^1\text{H}^\alpha_{i\text{-}1}$ and $^{13}\text{C}^\alpha_{i\text{-}1}$ with the chemical shift values of $^1\text{H}^\alpha_i$ and $^{13}\text{C}^\alpha_i$, (ii) using the chemical shift values of $^1\text{H}^\alpha_{i\text{-}1}$ and $^{13}\text{C}^\alpha_{i\text{-}1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of

the polypeptide chain and using the chemical shift values to locate secondary

structure elements (such as α-helices and β-sheets) within the polypeptide chain

(Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al.,

Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference

5   in their entirety).

[0090]   In one embodiment, the protein sample could, in addition to the RD

3D HA,CA,(CO),N,HN NMR experiment and the RD 3D HNNCAHA NMR

experiment, be further subjected to a RD 3D HNN<CO,CA> NMR experiment to

measure and connect the chemical shift values of a polypeptide backbone

10   carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, $^{13}C^{\alpha}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$. Then,

sequential assignments of the chemical shift value of $^{13}C'_{i-1}$, are obtained by

matching the chemical shift value of $^{13}C^{\alpha}_{i}$ measured by the RD 3D

HNN<CO,CA> NMR experiment with the sequentially assigned chemical shift

values of $^{13}C^{\alpha}$, ^{15}N, and $^{1}H^{N}$ measured by the RD 3D HA,CA,(CO),N,HN NMR

15   experiment and the RD 3D HNNCAHA NMR experiment.

[0091]   In another embodiment, the protein sample could, in addition to the

RD 3D HA,CA,(CO),N,HN NMR experiment and the RD 3D HNNCAHA NMR

experiment, be further subjected to (i) a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR

experiment to measure and connect the chemical shift values of the β-proton of

20   amino acid residue i, $^{1}H^{\beta}_{i}$, the β-carbon of amino acid residue i, $^{13}C^{\beta}_{i}$, the α-proton

of amino acid residue i, $^{1}H^{\alpha}_{i}$, the α-carbon of amino acid residue i, $^{13}C^{\alpha}_{i}$, and a

polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_{i}$, and (ii) a RD

3D HNN<CO,CA> NMR experiment to measure and connect the chemical shift

values of $^{13}C'_{i}$, the α-carbon of amino acid residue i+1, $^{13}C^{\alpha}_{i+1}$, the polypeptide

25   backbone amide nitrogen of amino acid residue i+1, $^{15}N_{i+1}$, and the polypeptide

backbone amide proton of amino acid residue i+1, $^{1}H^{N}_{i+1}$. Then, sequential

assignments are obtained by matching the chemical shift value of $^{13}C'_{i}$ measured

by the RD 3D HNN<CO,CA> NMR experiment with the chemical shift value of

$^{13}C'_{i}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment.

30   **[0092]**   In another embodiment, the protein sample could, in addition to the

RD 3D HA,CA,(CO),N,HN NMR experiment and the RD 3D HNNCAHA NMR

experiment, be further subjected to a RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment to measure and connect the chemical shift values of aliphatic protons (including α-, β-, and γ-protons) of amino acid residue i-1, $^1H^{ali}_{i-1}$, aliphatic carbons (including α-, β-, and γ-carbons) of amino acid residue i-1, $^{13}C^{ali}_{i-1}$, $^{15}N_i$,

5   and $^1H^N_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ for amino acid residues i having unique pairs of $^{15}N_i$ and $^1H^N_i$ chemical shift values are obtained by matching the chemical shift values of $^1H^\alpha$ and $^{13}C^\alpha$ measured by said RD 3D HNN$\underline{CAHA}$ NMR experiment and RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment with the chemical shift values of $^1H^\alpha_{i-1}$ and

10     $^{13}C^\alpha_{i-1}$ measured by said RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment and using the $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ chemical shift values to identify the type of amino acid residue i-1.

[0093]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR

15   experiment, be further subjected to a RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or a RD 3D $\underline{H},\underline{C},$C,H-TOCSY NMR experiment to measure and connect the chemical shift values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$ of amino acid residue i. Then, sequential assignments of the chemical shift values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$, the chemical shift values of a γ-proton, $^1H^\gamma_i$, and a γ-carbon, $^{13}C^\gamma_i$, in particular, are obtained by (i)

20   matching the chemical shift values of $^1H^\alpha_i$ and $^{13}C^\alpha_i$ measured using the RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or the RD 3D $\underline{H},\underline{C},$C,H-TOCSY RD NMR experiment with the chemical shift values of $^1H^\alpha_i$ and $^{13}C^\alpha$ measured by the RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment, the RD 3D HNN$\underline{CAHA}$ NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and (ii) using

25   the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$, including the chemical shift values of $^1H^\gamma_i$ and $^{13}C^\gamma_i$, to identify the type of amino acid residue i.

[0094]   In yet another embodiment, this method involves, in addition to the RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, further subjecting the protein sample to a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN

30   NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i-1, $^1H^\beta_{i-1}$, the β-carbon of amino acid residue i-1,

$^{13}C^{\beta}_{i-1}$, $^{1}H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{15}N_i$, and $^{1}H^{N}_i$. Then, sequential assignments of the chemical shift values of $^{1}H^{\beta}$ and $^{13}C^{\beta}$ are obtained by using the chemical shift values of $^{1}H^{\beta}_{i-1}$ and $^{13}C^{\beta}_{i-1}$ to identify the type of amino acid residue i-1.

[0095]　·　In another embodiment, the protein sample could, in addition to the RD 3D <u>HA</u>,<u>CA</u>,(CO),N,HN NMR experiment, the RD 3D HNN<u>CAHA</u> NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i, $^{1}H^{\beta}_i$, the β-carbon of amino acid residue i, $^{13}C^{\beta}_i$, $^{1}H^{\alpha}_i$, $^{13}C^{\alpha}_i$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$. Then, sequential assignments of the chemical shift value of $^{13}C'_i$ are obtained by matching the chemical shift values of $^{1}H^{\beta}_i$, $^{13}C^{\beta}_i$, $^{1}H^{\alpha}_i$, and $^{13}C^{\alpha}_i$ measured by the RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment with the sequentially assigned chemical shift values of $^{1}H^{\beta}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, and $^{1}H^{N}$ measured by the RD 3D <u>HA</u>,<u>CA</u>,(CO),N,HN NMR experiment, the RD 3D HNN<u>CAHA</u> NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment.

[0096]　In another embodiment, the protein sample could, in addition to the RD 3D <u>HA</u>,<u>CA</u>,(CO),N,HN NMR experiment, the RD 3D HNN<u>CAHA</u> NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_i$, $^{13}C^{\beta}_i$, $^{1}H^{\alpha}_i$, $^{13}C^{\alpha}_i$, $^{15}N_i$, and $^{1}H^{N}_i$. Then, sequential assignments are obtained by matching the chemical shift values of $^{1}H^{\beta}_i$, $^{13}C^{\beta}_i$, $^{1}H^{\alpha}_i$, and $^{13}C^{\alpha}_i$ with the chemical shift values of $^{1}H^{\beta}_{i-1}$, $^{13}C^{\beta}_{i-1}$, $^{1}H^{\alpha}_{i-1}$, and $^{13}C^{\alpha}_{i-1}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment.

[0097]　In another embodiment, the protein sample could, in addition to the RD 3D <u>HA</u>,<u>CA</u>,(CO),N,HN NMR experiment, the RD 3D HNN<u>CAHA</u> NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to measure and connect the chemical shift value of $^{13}C^{\beta}_i$, $^{13}C^{\alpha}_i$, $^{15}N_i$, and $^{1}H^{N}_i$. Then, sequential assignments are obtained by matching the chemical shift values of $^{13}C^{\beta}_i$ and $^{13}C^{\alpha}_i$ measured by

said 3D HNNCACB NMR experiment with the chemical shift values of $^{13}C^{\beta}_{i-1}$ and $^{13}C^{\alpha}_{i-1}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment.

[0098] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment, the RD 3D HNN$\underline{CAHA}$ NMR

5 experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, be further subjected to a RD 2D $\underline{HB},\underline{CB},$(CG,CD),HD NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_{i-1}$, $^{13}C^{\beta}_{i-1}$, and a δ-proton of amino acid residue I-1 with an aromatic side chain, $^{1}H^{\delta}_{i-1}$. Then, sequential assignments are obtained by matching (i) the chemical shift values of $^{1}H^{\beta}_{i-1}$ and $^{13}C^{\beta}_{i-1}$ measured

10 by said RD 2D $\underline{HB},\underline{CB},$(CG,CD),HD NMR experiment with the chemical shift values of $^{1}H^{\beta}$ and $^{13}C^{\beta}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, (ii) using the chemical shift values to identify amino acid residue i as having an aromatic side chain, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and

15 locating amino acid residues with aromatic side chains along the polypeptide chain.

[0099] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment, the RD 3D HNN$\underline{CAHA}$ NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, be further

20 subjected to a RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or a RD 3D $\underline{H},\underline{C},$C,H-TOCSY NMR experiment to measure and connect the chemical shift values of aliphatic protons (including α-, β-, and γ-protons) of amino acid residue i, $^{1}H^{ali}_{i}$, and aliphatic carbons (including α-, β-, and γ-carbons) of amino acid residue i, $^{13}C^{ali}_{i}$, of amino acid residue i. Then, sequential assignments of the chemical shift

25 values of $^{1}H^{ali}_{i}$ and $^{13}C^{ali}_{i}$, the chemical shift values of a γ-proton, $^{1}H^{\gamma}$, and a γ-carbon, $^{13}C^{\gamma}$, in particular, are obtained by (i) matching the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, and $^{13}C^{\alpha}_{i}$ measured using the RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or the RD 3D $\underline{H},\underline{C},$C,H-TOCSY RD NMR experiment with the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, and $^{13}C^{\alpha}_{i}$ measured by the RD 3D

30 $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment, the RD 3D HNN$\underline{CAHA}$ NMR experiment, and the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and (ii) using the chemical

shift values of $^1H^{ali}$ and $^{13}C^{ali}$, including the chemical shift values of $^1H^\gamma$ and $^{13}C^\gamma$, to identify the type of amino acid residue i.

[0100]   Yet another aspect of the present invention relates to a method for sequentially assigning chemical shift values of a β-proton, $^1H^\beta$, a β-carbon, $^{13}C^\beta$, an α-proton, $^1H^\alpha$, an α-carbon, $^{13}C^\alpha$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N{}_i$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1H^{\alpha/\beta}$ and $^{13}C^{\alpha/\beta}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i-1, $^1H^\beta{}_{i-1}$, the β-carbon of amino acid residue i-1, $^{13}C^\beta{}_{i-1}$, the α-proton of amino acid residue i-1, $^1H^\alpha{}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}C^\alpha{}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N{}_i$ and (2) a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i, $^1H^\beta{}_i$, the β-carbon of amino acid residue i, $^{13}C^\beta{}_i$, the α-proton of amino acid residue i, $^1H^\alpha{}_i$, the α-carbon of amino acid residue i, $^{13}C^\alpha{}_i$, $^{15}N_i$, and $^1H^N{}_i$. Then, sequential assignments of the chemical shift values of $^1H^\beta$, $^{13}C^\beta$, $^1H^\alpha$, $^{13}C^\alpha$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of the α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}{}_{i-1}$, and the chemical shift values of the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$, with $^1H^{\alpha/\beta}{}_i$ and $^{13}C^{\alpha/\beta}{}_i$, (ii) using $^1H^{\alpha/\beta}{}_{i-1}$ and $^{13}C^{\alpha/\beta}{}_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain (Spera et al., J. Am. Chem. Soc.,

113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0101] In one embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D $\underline{HA},\underline{CA}$,(CO),N,HN NMR experiment (i) to measure and connect chemical shift values of $^1H^{\alpha}_{i\text{-}1}$, $^{13}C^{\alpha}_{i\text{-}1}$, $^{15}N_i$, and $^1H^N_i$ and (ii) to distinguish between NMR signals for $^1H^{\alpha}/^{13}C^{\alpha}$ and $^1H^{\beta}/^{13}C^{\beta}$ measured in the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0102] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of $^1H^{\beta}_i$, $^{13}C^{\beta}_i$, $^1H^{\alpha}_i$, $^{13}C^{\alpha}_i$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$. Then, sequential assignments of the chemical shift value of $^{13}C'_i$ are obtained by matching the chemical shift values of $^1H^{\beta}_i$, $^{13}C^{\beta}_i$, $^1H^{\alpha}_i$, and $^{13}C^{\alpha}_i$ measured by the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment with the sequentially assigned chemical shift values of $^1H^{\beta}$, $^{13}C^{\beta}$, $^1H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, and $^1H^N$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0103] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to measure and connect the chemical shift values of a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i\text{-}1}$, $^{13}C^{\alpha}_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments of the chemical shift value of $^{13}C'_{i\text{-}1}$ are obtained by matching the chemical shift value of $^{13}C^{\alpha}_i$ measured by the RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment with the sequentially assigned chemical shift values of $^{13}C^{\alpha}$, ^{15}N, and $^1H^N$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0104] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to (i) a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}{}_{i}$, $^{13}C^{\beta}{}_{i}$, $^{1}H^{\alpha}{}_{i}$,

5 $^{13}C^{\alpha}{}_{i}$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'{}_{i}$ and (ii) a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to measure and connect the chemical shift values of $^{13}C'{}_{i}$, the α-carbon of amino acid residue $i+1$, $^{13}C^{\alpha}{}_{i+1}$, the polypeptide backbone amide nitrogen of amino acid residue $i+1$, $^{15}N_{i+1}$, and the polypeptide backbone amide proton of amino acid residue $i+1$, $^{1}H^{N}{}_{i+1}$. Then,

10 sequential assignments are obtained by matching the chemical shift value of $^{13}C'{}_{i}$ measured by said RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment with the chemical shift value of $^{13}C'{}_{i}$ measured by the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment.

[0105] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN

15 NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment to measure and connect the chemical shift values of $^{1}H^{ali}{}_{i-1}$, $^{13}C^{ali}{}_{i-1}$, $^{15}N_{i}$, and $^{1}H^{N}{}_{i}$. Then, sequential assignments of the chemical shift values of $^{1}H^{ali}{}_{i-1}$ and $^{13}C^{ali}{}_{i-1}$ for amino acid residues i having unique pairs of $^{15}N_{i}$ and $^{1}H^{N}{}_{i}$ chemical shift values are obtained by matching the chemical shift values of $^{1}H^{\beta}$,

20 $^{13}C^{\beta}$, $^{1}H^{\alpha}$, and $^{13}C^{\alpha}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment with the chemical shift values of $^{1}H^{\beta}{}_{i-1}$, $^{13}C^{\beta}{}_{i-1}$, $^{1}H^{\alpha}{}_{i-1}$, and $^{13}C^{\alpha}{}_{i-1}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and using the $^{1}H^{ali}{}_{i-1}$ and $^{13}C^{ali}{}_{i-1}$ chemical shift values to identify the type of amino acid residue $i-1$.

25 **[0106]** In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to measure and connect the chemical shift value of $^{13}C^{\beta}{}_{i}$, $^{13}C^{\alpha}{}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}{}_{i}$. Then, sequential assignments are obtained by matching the chemical shift values of $^{13}C^{\beta}{}_{i}$

30 and $^{13}C^{\alpha}{}_{i}$ measured by said 3D HNNCACB NMR experiment with the chemical

shift values of $^{13}C^{\beta}_{i-1}$ and $^{13}C^{\alpha}_{i-1}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}(CO)NHN$ NMR experiment.

[0107] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}(CO)NHN$ NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment, be further subjected to a RD 2D $\underline{HB},\underline{CB},(CG,CD),HD$ NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, and a δ-proton of amino acid residue i with an aromatic side chain, $^{1}H^{\delta}_{i}$. Then, sequential assignments are obtained by (i) matching the chemical shift values of $^{1}H^{\beta}_{i}$ and $^{13}C^{\beta}_{i}$ measured by said RD 2D $\underline{HB},\underline{CB},(CG,CD),HD$ NMR experiment with the chemical shift values of $^{1}H^{\beta}$ and $^{13}C^{\beta}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}(CO)NHN$ NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment, (ii) using the chemical shift values to identify amino acid residue i as having an aromatic side chain, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and locating amino acid residues with aromatic side chains along the polypeptide chain (Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0108] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}(CO)NHN$ NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C},C,H-COSY$ NMR experiment or a RD 3D $\underline{H},\underline{C},C,H-TOCSY$ NMR experiment to measure and connect the chemical shift values of aliphatic protons of amino acid residue i, $^{1}H^{ali}_{i}$, and aliphatic carbons of amino acid residue i, $^{13}C^{ali}_{i}$, of amino acid residue i. Then, sequential assignments of the chemical shift values of $^{1}H^{ali}_{i}$ and $^{13}C^{ali}_{i}$, the chemical shift values of a γ-proton, $^{1}H^{\gamma}_{i}$, and a γ-carbon, $^{13}C^{\gamma}_{i}$, in particular, are obtained by (i) matching the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, and $^{13}C^{\alpha}_{i}$ measured using the RD 3D $\underline{H},\underline{C},C,H-COSY$ NMR experiment or the RD 3D $\underline{H},\underline{C},C,H-TOCSY$ RD NMR experiment with the chemical shift values of $^{1}H^{\beta}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$, and $^{13}C^{\alpha}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}(CO)NHN$ NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta},N,HN$ NMR experiment, and (ii) using the chemical shift

values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$, including the chemical shift values of $^1H^\gamma_i$ and $^{13}C^\gamma_i$, to identify the type of amino acid residue i.

[0109]   A further aspect of the present invention involves a method for sequentially assigning the chemical shift values of aliphatic protons, $^1H^{ali}$, aliphatic carbons, $^{13}C^{ali}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D H̲,C̲,(C-TOCSY-CO),N,HN NMR experiment to measure and connect the chemical shift values of the aliphatic protons of amino acid residue i-1, $^1H^{ali}_{i-1}$, the aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a RD 3D H̲$^{\alpha/\beta}$,C̲$^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of the β-proton of amino acid residue i, $^1H^\beta_i$, the β-carbon of amino acid residue i, $^{13}C^\beta_i$, the α-proton of amino acid residue i, $^1H^\alpha_i$, the α-carbon of amino acid residue i, $^{13}C^\alpha_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}$, $^{13}C^{ali}$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of the α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$ and the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$ with the chemical shift values of $^1H^{\alpha/\beta}_i$ and $^{13}C^{\alpha/\beta}_i$ of amino acid residue i, (ii) using the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain (Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0110] In one embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, $^{13}C^{\alpha}_{i}$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_{i}$. Then, sequential assignments of the chemical shift value of $^{13}C'_{i}$ are obtained by matching the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, and $^{13}C^{\alpha}_{i}$ measured by the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment with the sequentially assigned chemical shift values of $^{1}H^{\beta}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, and $^{1}H^{N}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0111] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to measure and connect the chemical shift values of a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, $^{13}C^{\alpha}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$. Then, sequential assignments of the chemical shift value of $^{13}C'_{i-1}$ are obtained by matching the chemical shift value of $^{13}C^{\alpha}_{i}$ measured by the RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment with the sequentially assigned chemical shift values of $^{13}C^{\alpha}$, ^{15}N, and $^{1}H^{N}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0112] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to (i) a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, $^{1}H^{\alpha}_{i}$, $^{13}C^{\alpha}_{i}$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_{i}$, and (ii) a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to measure and connect the chemical shift values of $^{13}C'_{i}$, the α-carbon of amino acid residue i+1, $^{13}C^{\alpha}_{i+1}$, the polypeptide backbone amide nitrogen of amino acid residue i+1, $^{15}N_{i+1}$, and the polypeptide backbone amide proton of amino acid

residue i+1, $^{1}H^{N}_{i+1}$. Then, sequential assignments are obtained by matching the chemical shift value of $^{13}C'_{i}$ measured by the RD 3D HNN<CO,CA> NMR experiment with the chemical shift value of $^{13}C'_{i}$ measured by the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment.

5   **[0113]**   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment (i) to measure and connect the chemical shift values of $^{1}H^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha/\beta}_{i-1}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$, and (ii) to identify NMR signals for

10   $^{1}H^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha/\beta}_{i-1}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0114]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D

15   $\underline{HA},\underline{CA}$,(CO),N,HN NMR experiment (i) to measure and connect chemical shift values of $^{1}H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$ and (ii) to identify NMR signals for $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0115]   In another embodiment, the protein sample could, in addition to the
20   RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to measure and connect the chemical shift value of $^{13}C^{\beta}_{i}$, $^{13}C^{\alpha}_{i}$, $^{15}N_{i}$, and $^{1}H^{N}_{i}$. Then, sequential assignments are obtained by matching the chemical shift values of $^{13}C^{\beta}_{i}$ and $^{13}C^{\alpha}_{i}$ measured by said 3D HNNCACB NMR experiment

25   with the chemical shift values of $^{13}C^{\beta}_{i-1}$ and $^{13}C^{\alpha}_{i-1}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0116]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 2D

30   $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment to measure and connect the chemical shift values of $^{1}H^{\beta}_{i}$, $^{13}C^{\beta}_{i}$, and a δ-proton of amino acid residue i with an aromatic side

chain, $^1H^\delta_i$. Then, sequential assignments are obtained by matching the chemical shift values of $^1H^\beta_i$ and $^{13}C^\beta_i$ measured by said RD 2D <u>HB</u>,<u>CB</u>,(CG,CD)ND NMR experiment with the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ measured by the RD 3D <u>H</u>$^{\alpha/\beta}$,<u>C</u>$^{\alpha/\beta}$,N,HN NMR experiment and the RD 3D <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN

5 NMR experiment, using the chemical shift values to identify amino acid residue i as having an aromatic side chain, and mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and locating amino acid residues with aromatic side chains along the polypeptide chain.

10 **[0117]**   In another embodiment, the protein sample could, in addition to the RD 3D <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D <u>H</u>$^{\alpha/\beta}$,<u>C</u>$^{\alpha/\beta}$,N,HN NMR experiment, be further subjected to a RD 3D <u>H</u>,<u>C</u>,C,H-COSY NMR experiment or a RD 3D <u>H</u>,<u>C</u>,C,H-TOCSY NMR experiment to measure and connect the chemical shift values of aliphatic protons of amino acid

15 residue i, $^1H^{ali}_i$, and aliphatic carbons of amino acid residue i, $^{13}C^{ali}_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$, the chemical shift values of a γ-proton, $^1H^\gamma_i$, and a γ-carbon, $^{13}C^\gamma_i$, in particular, are obtained by (i) matching the chemical shift values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$ measured using the RD 3D <u>H</u>,<u>C</u>,C,H-COSY NMR experiment or the RD 3D <u>H</u>,<u>C</u>,C,H-

20 TOCSY NMR experiment with the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$ measured by the RD 3D <u>H</u>,<u>C</u>,(C-TOCSY-CO),N,HN NMR experiment and RD 3D <u>H</u>$^{\alpha/\beta}$,<u>C</u>$^{\alpha/\beta}$,N,HN NMR experiment, and (ii) using the chemical shift values of $^1H^{ali}_i$ and $^{13}C^{ali}_i$, including the chemical shift values of $^1H^\gamma_i$ and $^{13}C^\gamma_i$, to identify the type of amino acid residue i.

25 **[0118]**   The present invention also relates to a method for sequentially assigning chemical shift values of aliphatic protons, $^1H^{ali}$, aliphatic carbons, $^{13}C^{ali}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of reduced dimensionality (RD) nuclear magnetic

30 resonance (NMR) experiments on the protein sample, where the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$ which are encoded in peak pairs of a 3D NMR spectrum are detected in a phase sensitive manner, including: (1) a RD 3D <u>H</u>,<u>C</u>,(C-TOCSY-

CO),N,HN NMR experiment to measure and connect the chemical shift values of the aliphatic protons of amino acid residue i-1, $^1H^{ali}_{i-1}$, the aliphatic carbons of amino acid residue i-1, $^{13}C^{ali}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a RD 3D HNN<u>CAHA</u> NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i, $^1H^\alpha_i$, the α-carbon of amino acid residue i, $^{13}C^\alpha_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}$, $^{13}C^{ali}$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of the α-proton of amino acid residue i-1, $^1H^\alpha_{i-1}$ and the α-carbon of amino acid residue i-1, $^{13}C^\alpha_{i-1}$ with the chemical shift values of $^1H^\alpha_i$ and $^{13}C^\alpha_i$, (ii) using the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, <u>NMR of Proteins and Nucleic Acids,</u> Wiley, New York (1986); Grzesiek et al., <u>J. Biomol. NMR,</u> 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain (Spera et al., <u>J. Am. Chem. Soc.,</u> 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0119] In one embodiment, the protein sample could, in addition to the RD 3D <u>H,C,</u>(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN<u>CAHA</u> NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of a β-proton of amino acid residue i, $^1H^\beta_i$, a β-carbon of amino acid residue i, $^{13}C^\beta_i$, $^1H^\alpha_i$, $^{13}C^\alpha_i$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$. Then, sequential assignments of the chemical shift value of $^{13}C'_i$ are obtained by matching the chemical shift values of $^1H^\beta_i$, $^{13}C^\beta_i$, $^1H^\alpha_i$, and $^{13}C^\alpha_i$ measured by the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment with the sequentially assigned chemical shift values of $^1H^\beta$, $^{13}C^\beta$, $^1H^\alpha$, $^{13}C^\alpha$, ^{15}N, and $^1H^N$ measured by the RD 3D <u>H,C,</u>(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN<u>CAHA</u> NMR experiment.

[0120]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment to measure and connect the chemical shift values of a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, $^{13}C^{\alpha}_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments of the chemical shift value of $^{13}C'_{i-1}$ are obtained by matching the chemical shift value of $^{13}C^{\alpha}_i$ measured by the RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment with the sequentially assigned chemical shift values of $^{13}C^{\alpha}$, ^{15}N, and $^1H^N$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment.

[0121]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to (i) a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to measure and connect the chemical shift values of a β-proton of amino acid residue i, $^1H^{\beta}_i$, a β-carbon of amino acid residue i, $^{13}C^{\beta}_i$, the α-proton of amino acid residue i, $^1H^{\alpha}_i$, the α-carbon of amino acid residue i, $^{13}C^{\alpha}_i$, and a polypeptide backbone carbonyl carbon of amino acid residue i, $^{13}C'_i$, and (ii) a RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment to measure and connect the chemical shift values of $^{13}C'_i$, an α-carbon of amino acid residue i+1, $^{13}C^{\alpha}_{i+1}$, a polypeptide backbone amide nitrogen of amino acid residue i+1, $^{15}N_{i+1}$, and the polypeptide backbone amide proton of amino acid residue i+1, $^1H^N_{i+1}$. Then, sequential assignments are obtained by matching the chemical shift value of $^{13}C'_i$ measured by the RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment with the chemical shift value of $^{13}C'_i$ measured by the RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment.

[0122]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment (i) to measure and connect the chemical shift values of the α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$, the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$, $^{15}N_i$, and $^1H^N_i$, and (ii) to distinguish

NMR signals for the chemical shift values of $^1H^\beta_{i-1}$, $^{13}C^\beta_{i-1}$, $^1H^\alpha_{i-1}$, and $^{13}C^\alpha_{i-1}$ measured by the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment from NMR signals for the chemical shift values of $^1H^{ali}_{i-1}$ and $^{13}C^{ali}_{i-1}$ other than $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

5 **[0123]** In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment to measure and connect the chemical shift values of $^1H^\beta_i$, $^{13}C^\beta_i$, $^1H^\alpha_i$, $^{13}C^\alpha_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments are obtained by matching

10 the chemical shift values of $^1H^\beta_i$, $^{13}C^\beta_i$, $^1H^\alpha_i$, and $^{13}C^\alpha_i$ measured by said RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment with the chemical shift values of $^1H^\beta_{i-1}$, $^{13}C^\beta_{i-1}$, $^1H^\alpha_{i-1}$, and $^{13}C^\alpha_{i-1}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0124] In another embodiment, the protein sample could, in addition to the

15 RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to measure and connect the chemical shift values of $^{13}C^\beta_i$, $^{13}C^\alpha_i$, $^{15}N_i$, and $^1H^N_i$. Then, sequential assignments are obtained by matching the chemical shift values of $^{13}C^\beta_i$ and $^{13}C^\alpha_i$ measured by said 3D HNNCACB NMR experiment

20 with the chemical shift values of $^{13}C^\beta_{i-1}$ and $^{13}C^\alpha_{i-1}$ measured by the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0125] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a RD 2D

25 $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment to measure and connect the chemical shift values of $^1H^\beta_i$, $^{13}C^\beta_i$, and a δ-proton of amino acid residue i with an aromatic side chain, $^1H^\delta_i$. Then, sequential assignments are obtained by matching the chemical shift values of $^1H^\beta_i$ and $^{13}C^\beta_i$ measured by said RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment with the chemical shift values of $^1H^\beta$ and $^{13}C^\beta$ measured by the RD 3D

30 $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, using the chemical shift values to identify amino acid residue i as having an aromatic side chain, and mapping sets

of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and by locating amino acid residues with aromatic side chains along the polypeptide chain.

[0126] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or a RD 3D $\underline{H},\underline{C},$C,H-TOCSY NMR experiment to measure and connect the chemical shift values of aliphatic protons of amino acid residue i, $^1H^{ali}{}_i$, and aliphatic carbons of amino acid residue i, $^{13}C^{ali}{}_i$. Then, sequential assignments of the chemical shift values of $^1H^{ali}{}_i$ and $^{13}C^{ali}{}_i$, the chemical shift values of a γ-proton, $^1H^{\gamma}{}_i$, and a γ-carbon, $^{13}C^{\gamma}{}_i$, in particular, are obtained by (i) matching the chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$ measured using the RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment or the RD 3D $\underline{H},\underline{C},$C,H-TOCSY NMR experiment with the chemical shift values of $^1H^{\beta}{}_i$, $^{13}C^{\beta}{}_i$, $^1H^{\alpha}{}_i$, and $^{13}C^{\alpha}{}_i$ measured by the RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment and the RD 3D HNN$\underline{CAHA}$ NMR experiment, and (ii) using the chemical shift values of $^1H^{ali}{}_i$ and $^{13}C^{ali}{}_i$, including the chemical shift values of $^1H^{\gamma}{}_i$ and $^{13}C^{\gamma}{}_i$, to identify the type of amino acid residue i.

[0127] Another aspect of the present invention involves a method for obtaining nearly complete assignments of chemical shift values of ^1H, ^{13}C and ^{15}N of a protein molecule (excluding only chemical shift values of $^{13}C^{\delta}$ and $^{15}N^{\varepsilon 2}$ of glutamines, of $^{13}C^{\gamma}$ and $^{15}N^{\delta 2}$ of asparagines, of $^{13}C^{\varepsilon 3}$, $^1H^{\varepsilon 3}$, $^{13}C^{\zeta 2}$, $^1H^{\zeta 2}$, $^{13}C^{\zeta 3}$, $^1H^{\zeta 3}$, $^{13}C^{\eta 2}$, and $^1H^{\eta 2}$ groups of tryptophans, of $^{13}C^{\varepsilon}$ and $^1H^{\varepsilon}$ of methionines, and of labile sidechain protons that exchange rapidly with the protons of the solvent water) (Yamazaki et al., J. Am. Chem. Soc., 115:11054–11055 (1993), which is hereby incorporated by reference in its entirety), which are required for the determination of the tertiary structure of a protein in solution (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986), which is hereby incorporated by reference in its entirety). The method involves providing a protein sample and conducting four reduced dimensionality (RD) nuclear magnetic resonance (NMR) experiments on the protein sample, where the chemical shift values of ^1H and ^{13}C which are encoded in peak pairs of a 3D NMR

spectrum are detected in a phase sensitive manner and (1) a first experiment is selected from a RD three-dimensional (3D) $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, a RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment, or a RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment for obtaining sequential correlations of chemical shift values; (2) a second experiment is selected from a RD 3D HNN$\underline{CAHA}$ NMR experiment, a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, or a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment for obtaining intraresidue correlations of chemical shift values; (3) a third experiment is a RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment for obtaining assignments of aliphatic and aromatic sidechain chemical shift values; and (4) a fourth experiment is a RD 2D $\underline{HB},\underline{CB},$(CG,CD),HD NMR experiment for obtaining assignments of aromatic sidechain chemical shift values.

[0128]   In one embodiment of this method, the protein sample could be further subjected to a RD 2D $\underline{H},\underline{C},$H-COSY NMR experiment for obtaining assignments of aliphatic and aromatic sidechain chemical shift values.

[0129]   In another embodiment of this method, the first experiment is the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment and the second experiment is the RD 3D HNN$\underline{CAHA}$ NMR experiment.

[0130]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB},$(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{HA},\underline{CA},$(CO),N,HN NMR experiment to distinguish between NMR signals for $^1H^{\alpha}/^{13}C^{\alpha}$ and $^1H^{\beta}/^{13}C^{\beta}$ from the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment.

[0131]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C},$C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB},$(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C},$(C-TOCSY-CO),N,HN NMR experiment to obtain assignments of chemical shift values of $^1H^{ali}$ and $^{13}C^{ali}$.

[0132] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment to obtain assignments of chemical shift values of $^1H^\beta$ and $^{13}C^\beta$.

[0133] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0134] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0135] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment and a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of $^{13}C'$.

[0136] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN$\underline{CAHA}$ NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^1H and ^{13}C of aliphatic sidechains.

[0137] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN<u>CAHA</u> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D <u>HB</u>,<u>CB</u>,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^{1}H and ^{13}C of aromatic sidechains.

[0138] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment, RD 3D HNN<u>CAHA</u> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D <u>HB</u>,<u>CB</u>,(CG,CD),HD NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to obtain assignments of chemical shift values of ^{13}C$^\beta$.

[0139] In yet another embodiment of this method, the first experiment is the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the second experiment is the RD 3D HNN<u>CAHA</u> NMR experiment.

[0140] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<u>CAHA</u> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D <u>HB</u>,<u>CB</u>,(CG,CD),HD NMR experiment, be further subjected to a RD 3D <u>HA</u>,<u>CA</u>,(CO),N,HN NMR experiment to identify NMR signals for ^{1}H$^\alpha$/^{13}C$^\alpha$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0141] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<u>CAHA</u> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D <u>HB</u>,<u>CB</u>,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment to obtain assignments of chemical shift values of ^{1}H$^\beta$ and ^{13}C$^\beta$.

[0142] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<u>CAHA</u> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D <u>HB</u>,<u>CB</u>,(CG,CD),HD NMR experiment, be further subjected to a RD 3D

HNN<CO,CA> NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0143] In another embodiment, the protein sample could, in addition to the RD 3D H,C,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNNCAHA NMR experiment, RD 3D H,C,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $H^{\alpha/\beta},C^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0144] In another embodiment, the protein sample could, in addition to the RD 3D H,C,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNNCAHA NMR experiment, RD 3D H,C,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D HNN<CO,CA> NMR experiment and a RD 3D $H^{\alpha/\beta},C^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of $^{13}C'$.

[0145] In another embodiment, the protein sample could, in addition to the RD 3D H,C,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNNCAHA NMR experiment, RD 3D H,C,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D H,C,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^1H and ^{13}C of aliphatic sidechains.

[0146] In another embodiment, the protein sample could, in addition to the RD 3D H,C,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNNCAHA NMR experiment, RD 3D H,C,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D H,C,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^1H and ^{13}C of aromatic sidechains.

[0147] In another embodiment, the protein sample could, in addition to the RD 3D H,C,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNNCAHA NMR experiment, RD 3D H,C,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to obtain assignments of chemical shift values of $^{13}C^{\beta}$.

[0148] In yet another embodiment of this method, the first experiment is the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the second experiment is the RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment.

[0149] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{HA},\underline{CA}$,(CO),N,HN NMR experiment to identify NMR signals for $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0150] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment to identify NMR signals for $^{1}H^{\alpha/\beta}$ and $^{13}C^{\alpha/\beta}$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0151] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0152] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0153] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB},\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D

HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment and a RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of $^{13}C'$.

[0154] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H}$,$\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB}$,$\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}$,$\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^{1}H and ^{13}C of aliphatic sidechains.

[0155] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H}$,$\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB}$,$\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}$,$\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^{1}H and ^{13}C of aromatic sidechains.

[0156] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D $\underline{H}^{\alpha/\beta}$,$\underline{C}^{\alpha/\beta}$,N,HN NMR experiment, RD 3D $\underline{H}$,$\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB}$,$\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to obtain assignments of chemical shift values of $^{13}C^{\beta}$.

[0157] In yet another embodiment of this method, the first experiment is the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment and the second experiment is the RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment.

[0158] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment, RD 3D $\underline{H}$,$\underline{C}$,C,H-COSY NMR experiment, and RD 2D $\underline{HB}$,$\underline{CB}$,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{HA}$,$\underline{CA}$,(CO),N,HN NMR experiment to identify NMR signals for $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ in the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0159] In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H}$,$\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO}$,$\underline{CA}$> NMR experiment, RD 3D $\underline{H}$,$\underline{C}$,C,H-COSY NMR experiment, and RD 2D

HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN NMR experiment to identify NMR signals for $^{1}H^{\alpha/\beta}$ and $^{13}C^{\alpha/\beta}$ in the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment.

[0160]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H}^{\alpha/\beta},\underline{C}^{\alpha/\beta}$,CO,HA NMR experiment to obtain assignments of chemical shift values of polypeptide backbone carbonyl carbons, $^{13}C'$.

[0161]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^{1}H and ^{13}C of aliphatic sidechains.

[0162]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a RD 3D $\underline{H},\underline{C}$,C,H-TOCSY NMR experiment to obtain assignments of chemical shift values of ^{1}H and ^{13}C of aromatic sidechains.

[0163]   In another embodiment, the protein sample could, in addition to the RD 3D $\underline{H},\underline{C}$,(C-TOCSY-CO),N,HN NMR experiment, RD 3D HNN<$\underline{CO},\underline{CA}$> NMR experiment, RD 3D $\underline{H},\underline{C}$,C,H-COSY NMR experiment, and RD 2D HB,CB,(CG,CD),HD NMR experiment, be further subjected to a 3D HNNCACB NMR experiment to obtain assignments of chemical shift values of $^{13}C^{\beta}$.

[0164]   In addition, the above-described method for obtaining assignments of chemical shift values of ^{1}H, ^{13}C and ^{15}N of a protein molecule can involve further subjecting the protein sample to nuclear Overhauser effect spectroscopy (NOESY) (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986), which is hereby incorporated by reference in its entirety), to NMR

experiments that measure scalar coupling constants (Eberstadt et al., <u>Angew. Chem. Int. Ed. Engl.</u>, 34:1671-1695 (1995); Cordier et al., <u>J. Am. Chem. Soc.</u>, 121:1601-1602 (1999), which are hereby incorporated by reference in their entirety), or to NMR experiments that measure residual dipolar coupling constants (Prestegard, <u>Nature Struct. Biol.</u>, 5:517-522 (1998); Tjandra et al., <u>Science,</u> 278:1111-1114 (1997), which are hereby incorporated by reference in their entirety), to deduce the tertiary fold or tertiary structure of the protein molecule.

[0165]   A standard set of experiments (3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN, 3D HNN<u>CAHA</u>, 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA, 3D HNNCACB, 3D HNN<<u>CO,CA</u>>, 3D <u>HC</u>CH-COSY, 3D <u>HC</u>CH-TOCSY, 2D <u>HBCB</u>(CDCG)HD, and 2D ^{1}H-TOCSY-relayed <u>HCH</u>-COSY) can be employed for obtaining nearly complete resonance assignments of proteins including aliphatic and aromatic side chain spin systems.

[0166]   For larger proteins, complementary recording of highly sensitive 3D <u>HACA</u>(CO)NHN promises (i) to yield spin systems which escape detection in $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN, and (ii) to offer the distinction of α- and β-moiety resonances by comparison with $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN. Furthermore, employment of 50% random fractional protein deuteration (LeMaster, <u>Annu. Rev. Biophys. Biophys. Chem.</u>, 19:43–266 (1990); Nietlispach et al., <u>J. Am. Chem. Soc.</u>, 118:407–415 (1996); Shan et al., <u>J. Am. Chem. Soc.</u>, 118:6570–6579 (1996); Leiting et al., <u>Anal. Biochem.</u>, 265:351–355 (1998); Hochuli et al., <u>J. Biomol. NMR,</u> 17:33–42 (2000), which are hereby incorporated by reference in their entirety) in combination with the standard suite of NMR experiments (or transverse relaxation-optimized spectroscopy (TROSY) versions thereof) is attractive. The impact of deuteration for recording 4D $H^{\alpha/\beta}C^{\alpha/\beta}$(CO)NHN for proteins reorienting with correlation times up to around 20 ns (corresponding to a molecular weight around 30 kDa at ambient T) has been demonstrated (Nietlispach et al., <u>J. Am. Chem. Soc.</u>, 118:407–415 (1996) , which is hereby incorporated by reference in its entirety). Accordingly, 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN can be expected to maintain its pivotal role for obtaining complete resonance assignments for deuterated proteins at least up to about that size. Furthermore, protein deuteration offers the advantage that HNNCACB, which can be expected to become significantly less

sensitive than HNN<u>CAHA</u> for larger non-deuterated systems, (Szyperski et al., <u>J. Biomol. NMR,</u> 11:387–405 (1998), which is hereby incorporated by reference in its entirety) can be kept to recruit $^{13}C^{\beta}$ chemical shifts for sequential assignment (Shan et al., <u>J. Am. Chem. Soc.,</u> 118:6570–6579 (1996), which is hereby

5   incorporated by reference in its entirety).

[0167]   If solely chemical shifts are considered, the unambiguous identification of peak pairs is more involved whenever multiple peak pairs with degenerate chemical shifts in the other dimensions are present. The acquisition of the corresponding central peaks addresses this complication in a conceptually

10   straightforward fashion. However, it is important to note that pairs of peaks generated by a chemical shift splitting have quite similar intensity. In contrast, peak pairs arising from different moieties, possible located in different amino acid residues, most often do not show similar intensity. This is because the nuclear spin relaxation times, which determine the peak intensities, vary within each

15   residue as well as along the polypeptide chain. One may thus speak of a "nuclear spin relaxation time labeling" of peak pairs, which makes their identification an obvious task in most cases.

[0168]   Using cryogenic probes can reduce NMR measurement times by about a factor of 10 or more (Flynn et al., <u>J. Am Chem. Soc.,</u> 122:4823–4824

20   (2000), which is hereby incorporated by reference in its entirety). The high sensitivity of cryogenic probes shifts even the recording of RD NMR experiments entirely into the sampling limited data acquisition regime. In view of this dramatic reduction in spectrometer time demand, minimally achievable RD NMR measurement times are of keen interest to be able to adapt the NMR measurement

25   times to sensitivity requirements in future HTP endeavours. To further reduce the measurement time, and in view of the aforementioned "spin relaxation time labelling" of peak pairs, one may decide to also discard the use of ^{13}C-steady state magnetization for central peak detection.

[0169]   Although RD NMR was proposed in 1993 (Szyperski et al., <u>J. Biomol. NMR,</u> 3:127–132 (1993); Szyperski et al., <u>J. Am. Chem. Soc.,</u>

30   115:9307–9308 (1993), which are hereby incorporated by reference in their

entirety), its wide-spread use has been delayed by the more demanding spectral analysis when compared to conventional TR NMR. In particular, the necessity to extract chemical shifts from in-phase splittings suggests that strong computer support is key for employment of RD NMR on a routine basis. This can be readily addressed by using automated resonance assignment software for automated analysis of RD TR NMR data.

[0170] In conclusion, the joint employment of RD NMR spectroscopy (as well as phase-sensitively detected RD NMR spectroscopy), cryogenic probes, and automated backbone resonance assignment will allow one to determine a protein's backbone resonance assignments and secondary structure in a short time.

EXAMPLES

[0171] The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1 – Sample Preparation

[0172] NMR measurements were performed using a 1 mM solution of uniformly $^{13}C/^{15}N$ enriched "Z-domain" of the *Staphylococcal* protein A (Tashiro et al., J. Mol. Biol., 272:573–590 (1997); Lyons et al., Biochemistry, 32:7839–7845 (1993), which are hereby incorporated by reference in their entirety) dissolved in 90% D_2O/10% H_2O (20 mM K-PO$_4$) at pH = 6.5 and a 2 mM solution of $^{15}N/^{13}C$ doubly labeled ubiquitin in 90% H_2O/10% D_2O (50 mM K-PO$_4$; pH = 5.8).

Example 2 – NMR Spectroscopy

[0173] Multidimensional NMR experiments (Figure 2; Table 1) were recorded for a 1 mM solution of the 8.5 kDa Z-domain protein and/or a 2mM solution of ubiquitin at a temperature of 25 °C. The spectra were assigned, and the chemical shifts obtained from RD NMR were in very good agreement with those previously determined at 30 °C using conventional triple resonance (TR)

NMR spectroscopy (Tashiro et al., J. Mol. Biol., 272:573–590 (1997); Lyons et al., Biochemistry, 32:7839–7845 (1993), which are hereby incorporated by reference in their entirety).

[0174] NMR experiments were recorded at a temperature of 25°C on a Varian Inova 600 spectrometer equipped with a new generation ^{1}H$\{^{13}$C,^{15}N$\}$ triple resonance probe which exhibits a signal-to-noise ratio of 1200:1 for a standard 0.1% ethylbenzene sample.

[0175] Specific embodiments of the 11 RD NMR experiments disclosed by the present invention were implemented for the present study. Figure 2 provides a survey of (i) the names, (ii) the magnetization transfer pathways and (iii) the peak patterns observed in the projected dimension of each of the 11 RD NMR experiments disclosed by the present invention. For simplicity, Figure 2 shows the in-phase peak pattern from the cosine-modulated RD NMR experiments. When acquiring the sine-modulated congeners, anti-phase peak pairs were observed instead (see Figure 1). The group comprising the first three experiments are designed to yield "sequential" connectivities via one-bond scalar couplings: 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN (Figure 2A), 3D HACA(CO)NHN (Figure 2B), and 3D HC(C-TOCSY-CO)NHN (Figure 2C). The following three experiments provide "intraresidual" connectivities via one-bond scalar couplings: 3D HNNCAHA (Figure 2D), 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA (Figure 2E), and 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN (Figure 2F). 3D HNN<CO,CA> (Figure 2G) offers both intraresidual ^{1}H^N–^{13}C$^\alpha$ and sequential ^{1}H^N–^{13}C' connectivities. Although 3D HNNCAHA (Figure 2D), 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$NHN (Figure 2F) and 3D HNN<CO,CA> (Figure 2G) also provide sequential connectivities via two-bond ^{13}C$^\alpha_{i-1}$–^{15}N$_i$ scalar couplings, those are usually smaller than the one-bond couplings (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety), and obtaining complete backbone resonance assignments critically depends on experiments designed to provide sequential connectivities via one-bond couplings (Figures 2D-F). 3D HCCH-COSY (Figure 2H) and 3D HCCH-TOCSY (Figure 2I) allow one to obtain assignments for the "aliphatic" side chain spin systems, while 2D HBCB(CDCG)HD (Figure 2J) and

2D ^{1}H-TOCSY-relayed HCH-COSY (Figure 2K) provide the corresponding information for the "aromatic" spin systems.

[0176]   The RD NMR experiments are grouped accordingly in Table 1, which lists for each experiment (i) the nuclei for which the chemical shifts are measured, (ii) if and how the central peaks are acquired and (iii) additional notable technical features. State-of-the art implementations (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996); Kay, J. Am. Chem. Soc., 115:2055–2057 (1993); Grzesiek et al., J. Magn. Reson., 99:201–207 (1992); Montelione et al., J. Am. Chem. Soc., 114:10974–10975 (1992); Boucher et al., J. Biomol. NMR, 2:631–637 (1992); Yamazaki et al., J. Am. Chem. Soc., 115:11054–11055 (1993); Zerbe et al., J. Biomol. NMR, 7:99–106 (1996); Grzesiek et al., J. Biomol. NMR, 3:185–204 (1993), which are hereby incorporated by reference in their entirety) making use of pulsed field z-gradients for coherence selection and/or rejection, and sensitivity enhancement (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) were chosen, which allow executing these experiments with a single transient per acquired free induction decay (FID). Semi (Grzesiek et al., J. Biomol. NMR, 3:185–204 (1993), which is hereby incorporated by reference in its entirety) constant-time (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego, (1996), which is hereby incorporated by reference in its entirety) chemical shift frequency-labeling modules were used throughout in the indirect dimensions in order to minimize losses arising from transverse nuclear spin relaxation. Figures 3A-K provide comprehensive descriptions of the RD NMR r.f. pulse sequences.

[0177]   In total, nine RD TR NMR experiments were recorded for the Z-domain protein (Table 2) and eleven RD TR NMR experiments for ubiquitin (Table 3). Figures 5-15 show composite plots of [ω_1,ω_3]- or [ω_1,ω_2]-strips taken from the recorded phase-sensitively detected RD NMR experiments. Except for 3D HNNCAHA, 3D HNN<CO,CA> and 2D ^{1}H-TOCSY-relayed HCH-COSY (Figure 1), central peaks were derived from ^{13}C magnetization (Figure 2; Table 1). Hence, two subspectra, I and II containing the peak pairs and central peaks respectively, were generated (Szyperski et al., J. Am. Chem. Soc., 118:8146–8147

(1996); Szyperski et al., J. Biomol. NMR, 11:387–405 (1998), which are hereby incorporated by reference in their entirety) for eight of the RD NMR experiments (Figure 2).

Table 2. Phase-Sensitively Detected RD NMR Data Sets Recorded for "Z-domain" Protein

	3D HACA(CO)NHN	3D HNNCAHA	3D H$^{\alpha\beta}$C$^{\alpha\beta}$(CO)NHN	3D H$^{\alpha\beta}$C$^{\alpha\beta}$NHN	3D HNN<CO,CA>	3D HC(C-TOCSY-CO)NHN	3D HCCH-COSY	3D HCCH-TOCSY	2D HBCB(CGCD)HD
^{1}H Resonance Frequency (MHz)	600	600	600	600	600	600	600	600	600
No. of Points[a]									
collected	40,28,512	64,28,512	76,28,512	68,28,512	56,28,512	76,28,512	90,20,12	80,20,512	76,512
after LP	80,56,512	96,56,512	152,56,512	136,56,512	112,56,512	152,56,512	180,40,512	160,40,512	152,512
after zero filling	256,64,1024	256,64,1024	256,64,1024	256,64,1024	256,64,1024	256,64,1024	256,64,1024	256,64,1024	512,1024
window functions[b]	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/70/90	sin 70/90
No. of Transients[c]	2	2	2	2	2	2	2	2	64
Spectral Width[d] ($\omega_1, \omega_2, \omega_3, \omega_4$; Hz)	6000 (RD), 1300, 6983	5200.2 (RD), 1300, 6983	12001.2 (RD), 1300, 6983	12001.2 (RD), 1300, 6983	8000 (RD), 1300, 6983	12001.2 (RD), 1300, 6983	12001.2 (RD), 3125, 6983	12001.2 (RD), 3125, 6983	12001.2 (RD), 6983
t_{max} (ms)[e]	6.5, 20.77, 73.18	12.11, 20.77, 73.18	6.25, 20.77, 73.18	5.58, 20.77, 73.18	6.88, 20.77, 73.18	6.25, 20.77, 73.18	7.42, 6.08, 73.18	6.58, 6.08, 73.18	6.25, 73.18
Carrier Position ($\omega_1, \omega_2, \omega_3, \omega_4$; ppm)[f]	53.05 (3.78), 119.3, 4.78	53.05 (3.78), 119.3, 4.78	38.05 (3.78), 119.3, 4.78	38.05 (3.78), 119.3, 4.78	171.07 (53.0), 119.3, 4.78	38.05 (3.78), 119.3, 4.78	38.05 (3.78), 38.05, 4.78	38.05 (3.78), 38.05, 4.78	33.04(3.78), 4.78
Recycle Delay (s)[g]	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.77	0.72
Collection Time (h)[h]	6.7	10.73	12.74	11.4	9.38	12.74	10.77	11.0	7.82

a "No. of Points" represents the number of complex data points used to sample indirect dimensions. Before Fourier transformation, the time domain points are extended by linear prediction (LP).

b The "Window Function" is a mathematical function multiplied with the FID along each indirect dimension before zero-filling and Fourier transformation.

c "No. of Transients" represents the number of FIDs acquired for each real increment.

d "Spectral Width" is the frequency range covered in each dimension.

e "t_{max}" is the maximum chemical shift evolution time.

f "Carrier Position" refers to the frequency (in ppm) of the center point of the spectrum along each dimension.

g "Recycle Delay" denotes the relaxation delay between acquisitions of FIDs.

h "Collection Time" is the total measurement time.

Table 3. Phase-Sensitively Detected RD NMR Data Sets Recorded for Ubiquitin

	3D HACA(CO)NHN	3D HNNCAHA	3D H$^{α/β}$C$^{α/β}$(CO)NHN	3D H$^{α/β}$C$^{α/β}$NHN	3D HNN<CO,CA>	3D H$^{α/β}$C$^{α/β}$ COHA	3D HCC-TOCSY-CONHN	3D HCCH-COSY	3D HCCH-TOCSY	2D HBCB(CGCD)HD	2D H-TOCSY-HCH-COSY
^{1}H Resonance Frequency (MHz)	600	600	600	600	600	600	600	600	600	600	600
No. of Points^a											
collected	40,28,512	56,28,512	76,28,512	68,28,512	56,28,512	76,32,512	76,28,512	90,20,512	80,20,512	76,512	100,512
after LP						152,64,512				152,512	150,512
after zero filling						256,64,1024				512,1024	512,1024
window functions^b						sin 70/70/90				sin 70/90	sin 70/90
No. of Transients^c	2	2	2	2	2	2	2	2	2	32	16
Spectral Width^d ($ω_1, ω_2, ω_3, ω_4$; Hz)	6000 (RD), 1337, 7018	6000 (RD), 1337, 7018	12001.2 (RD), 1337, 7018	12001.2 (RD), 1337, 7018	8000 (RD), 1337, 7018	12001(RD), 1800, 7018	12001.2 (RD), 1337, 7018	12001.2 (RD), 3125, 7018	12001.2 (RD), 3125, 7018	12001(RD), 7018	10000(RD), 7018
t_{max} (ms)^e	6.5, 20.2, 72.8	9.17, 20.2, 72.8	6.25, 20.2, 72.8	5.58, 20.2, 72.8	6.88, 20.2, 72.8	6.25, 17.2, 72.8	6.25, 20.2, 72.8	7.42, 6.08, 72.8	6.58, 6.08, 72.8	6.25, 72.8	9.9, 72.8
Carrier Position ($ω_1, ω_2, ω_3, ω_4$; ppm)^f	53.04 (3.78), 119.3, 4.78	53.04 (3.78), 119.3, 4.78	38.05 (3.78), 119.3, 4.78	38.05 (3.78), 119.3, 4.78	171.07 (53.0), 119.3, 4.78	38.05 (3.78), 172.4, 4.78	38.05 (3.78), 119.3, 4.78	38.05 (3.78), 38.05, 4.78	38.05 (3.78), 38.05, 4.78	33.05 (3.78), 4.78	120.05 (4.78), 4.78
Recycle Delay (s)^g	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.77	0.72	0.77
Collection Time (h)^h	6.7	9.38	12.73	11.39	9.38	14.55	12.73	10.77	10.99	3.91	1.37

a   "No. of Points" represents the number of complex data points used to sample indirect dimensions. Before Fourier transformation, the time domain points are extended by linear prediction (LP).
b   The "Window Function" is a mathematical function multiplied with the FID along each indirect dimension before zero-filling and Fourier transformation.
c   "No. of Transients" represents the number of FIDs acquired for each real increment.
d   "Spectral Width" is the frequency range covered in each dimension.
e   "t_{max}" is the maximum chemical shift evolution time.
f   "Carrier Position" refers to the frequency (in ppm) of the center point of the spectrum along each dimension.
g   "Recycle Delay" denotes the relaxation delay between acquisitions of FIDs.
h   "Collection Time" is the total measurement time.

Example 3 - HTP Assignment Strategy

[0178] The comprehensive analysis of the suite of multidimensional spectra recorded for the present study (Tables 1-3) lays the foundation to devise strategies for RD NMR-based HTP resonance assignment of proteins.

5   **[0179]** For proteins in the molecular weight range up to about 20 kDa, 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN plays a pivotal role. Firstly, the peak patterns observed along $\omega_1(^{13}C^{\alpha/\beta})$ in subspectra I and II enable sequential resonance assignment in combination with HNN<u>CAHA</u> and HNNCACB, respectively, by matching intraresidue and sequential $^1H^{\alpha}$, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts. (When considering

10   "nuclear spin relaxation time labelling" of peak pairs, subspectrum II derived from ^{13}C steady state magnetization provides largely redundant information when compared with subspectrum I. However, the observation of the central peaks allows direct matching of peak positions between subspectrum II, essentially a CBCA(CO)NHN spectrum, and HNNCACB.) Moreover, this set of chemical

15   shifts alone provides valuable information for amino acid type identification (Zimmerman et al., <u>J. Mol. Biol.</u>, 269:592–610 (1997); Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego, (1996); Grzesiek et al., <u>J. Biomol. NMR</u>, 3:185–204 (1993), which are hereby incorporated by reference in their entirety). Complementary recording of 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$COHA and 3D

20   HNN<<u>CO,CA</u>> contributes polypeptide backbone $^{13}C=O$ chemical shift measurements for establishing sequential assignments: the intraresidue correlation is obtained by $\omega_1(^{13}C^{\alpha/\beta})$ peak pattern matching with 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN, and the sequential correlation is inferred from $^{13}C^{\alpha}$, ^{15}N and $^1H^N$ chemical shifts in 3D HNN<<u>CO,CA</u>> (Szyperski et al., <u>J. Biomol. NMR</u>, 11:387–405 (1998), which is

25   hereby incorporated by reference in its entirety). Notably, even for medium-sized (non-deuterated) proteins this approach is superior to the use of a low sensitivity HNN<u>CACO</u>-type experiment (*e.g.*, in combination with HNN<u>COCA</u>), where the magnetization transfer *via* rapidly relaxing $^{13}C^{\alpha}$ relies on the rather small $^{15}N-^{13}C^{\alpha}$ one-bond scalar coupling. Secondly, comparison of $\omega_1(^{13}C^{\alpha/\beta})$ peak

30   patterns with 3D H<u>CC</u>H-COSY and TOCSY connects the $C^{\alpha/\beta}/H^{\alpha/\beta}$ chemical shifts with those of the aliphatic side chain spin systems (For Z-domain, complete

side chain assignments were obtained for all but six residues using 3D $\underline{\text{HCCH}}$-COSY only.), while comparison of $\omega_1(^{13}C^\beta)$ peaks with 2D $\underline{\text{HBCB}}$(CDCG)HD and subsequent linking with $^1H^\delta$ chemical shifts detected in 2D ^{1}H-TOCSY-relayed $\underline{\text{HCH}}$-COSY affords assignment of the aromatic spin systems. Since for

5   many amino acid residues the two β-protons exhibit non-degenerate chemical shifts, the connection of $\underline{\text{H}}^{\alpha/\beta}\underline{\text{C}}^{\alpha/\beta}$(CO)NHN and $\underline{\text{HBCB}}$(CDCG)HD or $\underline{\text{HCCH}}$-COSY/TOCSY may in fact often rely on comparison of three chemical shifts, *i.e.*, $\delta(^1H^{\beta 2})$, $\delta(^1H^{\beta 3})$ and $\delta(^{13}C^\beta)$. This consideration underscores the potential of recruiting β-proton chemical shifts for establishing sequential resonance

10   assignments.

[0180]   Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the

15   scope of the invention as defined in the claims which follow.